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CELL CULTURE TECHNOLOGY FOR PHARMACEUTICAL AND CELL-BASED THERAPIES

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Sadettin S. Ozturk
Wei-Shou Hu

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Validation of Cell Culture-Based Processes and Qualification of Associated Equipment and Facility

Chandra M. Dwivedi*
Bayer Corporation, Berkeley, California, U.S.A.

INTRODUCTION

Why validate? Though validation is a well-accepted and recognized cGMP requirement in today's Pharma business, this question is often posed during the product or process development (PD) activities in a start-up or even in an established company. In a nutshell, validation is not only a regulatory requirement, but it makes "good business sense." Validated processes assure production of quality product, batch after batch, and ultimately result in fewer headaches down the road in terms of fewer deviations during production, quality assurance (QA) discrepancy investigations, adverse events from the field, and regulatory observations (483s and its global equivalent) during regulatory inspections. In addition, they improve cost effectiveness in terms of preventing process failures, lot rejections, re-processing of salvageable lots, and attaining maximum plant capacity. Moreover, a sound and thorough validation strategy not only assures the production of top quality products, but also builds confidence and provides peace of mind to its customers. It also boosts the morale of the company employees and help build a sound and trustworthy relationship and track record with the regulatory agencies. The latter may come as a blessing for a company's future dealings with the regulatory agencies.

The term process validation originated in 1983 when the Food and Drug Administration (FDA) expanded the cGMP guidelines to cover demonstration of process consistency/reproducibility, but the guidelines were not finalized until 1987 (1). These guidelines were originally intended to be adopted by all drug product and biological manufacturers, but were later extended to the medical device and diagnostic manufacturers and to the blood collection/distribution/users and blood product manufacturers (2,3). Though originally intended only for the finished drug

*Currently at BIOGEN IDEC, Inc., Cambridge, MA, U.S.A.

product, these regulations have been recently extended to bulk drugs and bulk biologicals (4).

The original definition of the term process validation was described by the FDA as ''Establishing a documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its predetermined specifications and quality attributes.''

In practice, process validation (process performance qualification, PPQ) is more complicated than the simple definition stated above and is only one element of the overall validation process. It is a culmination of all other validation studies, such as equipment qualification (installation qualification, IQ; operational qualification, OQ; and performance qualification, PQ), computer qualification (IQ and OQ), utilities and facilities qualification (IQ, OQ, and PQ) cleaning validation (PQ), environmental qualification (PQ), and analytical qualification (PQ), all covered under a validation master plan (VMP) written for each new technology, process, or a product.

The invention of recombinant DNA technology in the late 1970s and its widespread application to eukaryotic and prokaryotic cells for developing unique medical applications/treatments resulted in the establishment of a new field known as ''Genetic Engineering'' today (5,6). These developments opened the floodgates for innovation that resulted in the establishment of many biotechnology companies worldwide. Of these, more than 50% of the biotechnology companies are working on cell culture technology for producing pharmaceutical and cellular therapies.

Due to continued innovation in this field the application of process validation concepts and guidelines are becoming increasingly complex, challenging, and difficult to understand by technical professionals, regulatory auditors, and cGMP compliance enforcers working in the pharmaceutical and biotechnology-related organizations. Since it is impossible to cover all aspects of process validation for the numerous biotechnology-derived products in this chapter, an attempt will be made only to provide a simplified version of the regulatory requirements that are needed for licensing cell culture-derived pharmaceuticals and cellular therapies. This chapter is intended to provide a bird's-eye view of the regulatory requirements for process validation to entrepreneurs before they plan for building a new manufacturing plant and expect to obtain licensure for a product (Product License Application, PLA) or a biologic (Biologic License Application, BLA) or a drug (New Drug Application, NDA) from regulatory agencies. This chapter is expected to prepare them well before they begin that challenging, eventful, exhausting, memorable, and ultimately rewarding journey.

APPROACH AND RATIONALE

The innumerable amount of research and development studies conducted on a large number of medical products has enabled us to understand that the quality attributes for any given product are not an unexpected output. But, are largely dependent on the process parameters used during their production. Therefore, the control of quality attributes for any biological or pharmaceutical product is in our hands; and with the development of new technologies, quality attributes for the new products can now be built into the manufacturing process. In this respect, the process design in relation to the respective product quality attributes has become crucial for the development and licensing of the medical and pharmaceutical products (7–10).

Since the breakthrough in genetic engineering a few decades ago, numerous medical, biological, pharmaceutical, and diagnostic products and applications

based on cell culture technology have been invented. They are based on microbial fermentation (eukaryotic and prokaryotic), hybridoma technology, and tissue regeneration. Even plant cell technology is being evaluated to produce medical and therapeutic products for human use. The examples of the cells used for this purpose are: bacteria (*Escherichia coli*), fungi (*Aspergillus*, *Saccharomyces*), mammalian cells (CHO, BHK, myeloma, melanoma, hybridoma, etc.), insect cells (*Drosophila*), and plant cells (tobacco, spinach, etc.). The majority of the products are secreted by the cells in the spent medium (harvest) by applying the rDNA technologies and manipulation of respective genes in the cells. The examples of recombinant products derived from these technologies are: erythropoietin (rEPO), anti-hemophilic factor (rFVIII), tissue plasminogen activator (rTPA), growth factors (EGF, TGF, PDGF, TNF, etc.), hormones (Insulin, LH, FSH, etc.), interferons (IF-1, IF-2, etc.), interleukins (IL-2, IL-4, IL-6, etc.), monoclonal antibodies (mAbs), and other enzymes and proteins (cerezyme, galactosidase, etc.). Some of the products are expressed in the inclusion bodies within cells and the cells therefore must be lysed to extract the products out (insulin, EGF, etc.). Epithelial cells, neuroblastoma, osteoblastoma and cartilage cells are being grown in laboratories and used as medical devices for a number of treatments (burns, tissue implant, tissue regeneration, etc.). A number of monoclonal antibodies are being generated from bacterial, mammalian, and plant cell technologies for the treatment of cancer, autoimmune diseases, and other immunological disorders.

A general approach to streamline validation concepts and policies has been evolving over the last number of years. These efforts have resulted in better understanding of the requirements for the validation by the industry professionals. For the purposes of clarity and better understanding this article will employ the newly emerging approach on validation concepts (11,12). Accordingly, qualification of all equipment and systems (design qualification, DQ; installation qualification, IQ; operational qualification, OQ; and performance qualification, PQ) will be referred as ''Equipment Qualification'' and not as ''Validation.'' The term ''Validation'' will be used only for ''Process Validation'' studies that are related with the studies (with or without active ingredient) at the small-scale (lab-scale) or full production scale (process validation, PV or PPQ).

The variety of cell culture technologies and many different approaches to use them as pharmaceutical products or medical devices makes the task of building the quality attributes in the manufacturing process very challenging. This also makes the task of process validation more difficult as generic models of process validation cannot be used, and every process validation study needs to be devised from scratch based on the technology being used. For example, the level of impurities (DNA, host cell contaminating proteins, etc.) may be substantially less in the starting material where the product is secreted out in the spent medium (harvest) as compared to the product that is expressed intracellularly such as in the inclusion bodies. Therefore, the design of the manufacturing process and the resultant process validation studies would be very different for the two approaches to isolate and purify the product(s).

The possible impurities and contaminants in a cell culture-based product are: intact cells, adventitious agents [bacteria, fungi, mycoplasma, viruses, transmissible spongiform encephalitis (TSE)/bovine spongiform encephalitis (BSE)], endogenous retroviruses, host cell nucleic acids and proteins, foreign proteins (from raw materials and microbial contaminations), endotoxins, and contaminating process chemicals (13). A validated process, therefore, must demonstrate effective removal, inactivation, or reduction of these impurities and contaminants to acceptable levels.

Though it is preferable to perform process validation studies at full-scale operational level, it is not always possible to perform them at manufacturing scale due to practical limitations (e.g., virus and nucleic acid reduction studies may require huge amounts of model viruses and nucleic acids). In such cases, scaled-down bench-level studies are acceptable as long as all process input parameters are kept the same as in the full-scale and the output parameters are comparable to the full-scale (14). Whenever this approach is used, demonstration and justification of the acceptability of the scaled-down model should be performed prior to formal process validation.

PROCESS DEVELOPMENT

Development of a Defined Process

The critical steps for the development of a defined process are outlined in Fig. 1. We will examine below the requirements for developing a reliable and reproducible process for a cell culture derived product. The definition of a defined process may be summarized as "a process that provides a high degree of assurance that it will consistently produce a product meeting its predetermined specifications and quality attributes." This definition seems simple and doable (in the beginning phase of a project) but becomes difficult to achieve when all the details for a cell culture-based product are brought into consideration. Adequate confidence must be built by doing sufficient experimentation and development work to demonstrate that the process can consistently produce a product of pre-specified quality. Range finding (feed stream) studies should be performed for every critical and noncritical process parameter (15), and operational set-points must be established after completion of the range finding studies. Worst-case studies (upper and lower ranges) should be performed during the development phase, (as it is much easier to do them during development than during actual production). Alert and action levels (limits) for out-put parameters (test results and specifications) must be established with adequate justification. In-process and final product specifications (acceptance criteria) must be defined clearly with sound scientific justifications.

The success of a well executed project depends on a well written process development (PD) report with sufficient details for every aspect of the process and a well executed transfer of technology from the R&D department to the operations department. The R&D personnel not only adequately transfer the technology, but must provide training to production personnel in every aspect of the process. The role of the R&D personnel does not end here, they should actively monitor the process after successful process validation by applying the statistical tools such as statistical process control. Post-validation process data must be analyzed to ensure that the process performs within the established boundaries. Process capability (Cpk) calculations must be performed on the post-validation process data to evaluate process performance. The process data should also be analyzed by applying other statistical tests, such as Student's *t*-test, to determine confidence intervals on process performance. A 95% confidence interval is generally acceptable for process validation studies. Many companies, however, run their production processes at 98% confidence interval or up to ± 6 SD of the validated process parameters. These analyses demonstrate whether the process is in control and build confidence for running the process on a consistent basis.

The importance and relevance of good PD work that eventually pays off many fold must be emphasized here. It is generally acknowledged that many pharmaceutical

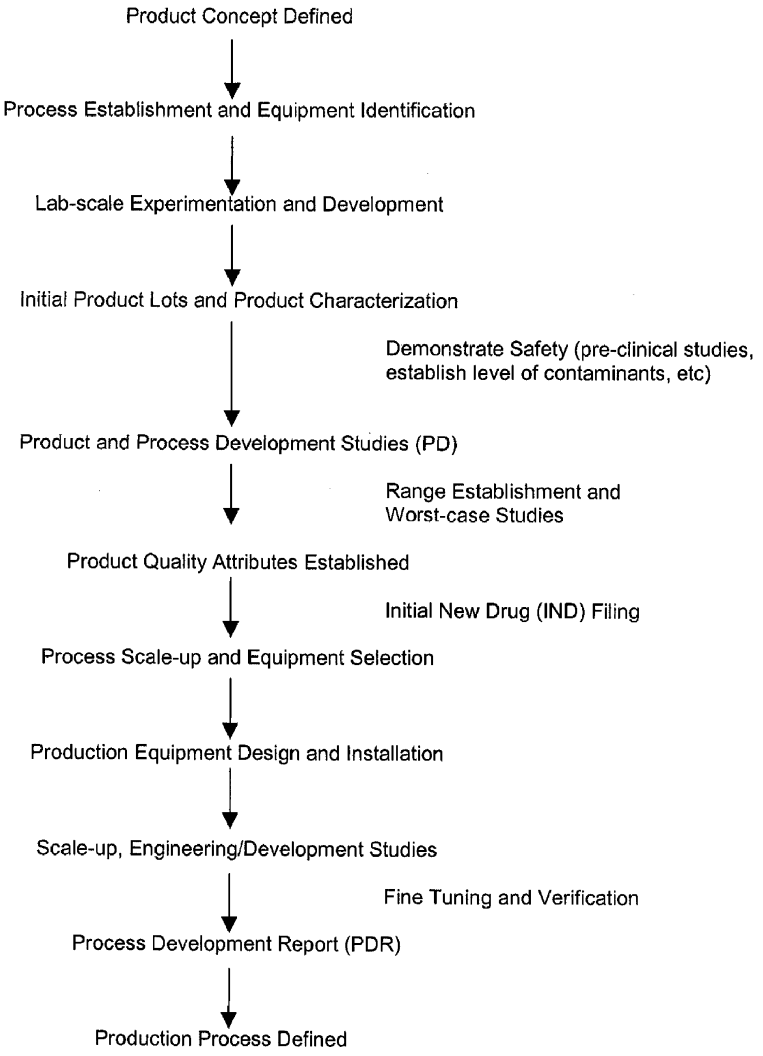

Figure 1 Critical steps for developing a defined process.

and biotechnology organizations shy away from doing comprehensive PD work as they are in a rush to reach the marketplace. In our competitive world of today, timing is key for making or breaking of an organization. Often what we do not realize is that there are no short cuts and eventually (sooner or later) we have to do the required PD work. The smart approach, therefore, would be to perform all required PD work before process validation, rather than during process validation or after completion of a process validation project. In the latter case, the validation projects generally become confusing, cost a great deal of money, and delay project completion (16).

A poorly developed process will typically allow only narrow ranges for operational parameters and may result in the rejection of large amounts of otherwise good

in-process material produced slightly outside the narrow process ranges developed. Extension of the process ranges or scale-up of manufacturing processes after initial validation requires time consuming regulatory review and approvals, repetition of all the work performed previously, and almost always turns into a costly validation project. It prevents pioneering organizations from taking leadership positions in the marketplace due to limited product supplies. It may lead and encourage competitors to enter the field and snatch the leadership position from the organizations that developed the product at the first place. It is a lesson many organizations learn, albeit late.

Process Development Report

The importance of well-executed PD work and a well-written process development report (PDR) cannot be emphasized enough. PD and PDRs are the key components of a successful technology transfer from R&D to manufacturing (8). The success or failure of a process validation project greatly depends on the quality and details of the PD work performed and the quality of PDRs in terms of their content, clarity, and completeness. Poorly written reports often cause a great deal of frustration for all involved, result in unnecessary delays, impact project schedule, and even lead to ultimate failure of a project. Many organizations perform excellent PD work, but lack in writing clear and complete reports. Ideal PDRs should contain the following information in as much detail as possible:

- Objective and definition of a process/product
- Scope and rationale
- Process description
- Process flow chart
- Materials and methods
- Equipment and facilities
- Utilities and accessories
- HVAC and environmental requirements
- Process input and output parameters (critical and noncritical)
- In-process testing and acceptance criteria
- Product specifications
- Calibration and preventive maintenance
- Other process requirements
- Result and discussion
- Conclusion
- References

Process Parameters

It is paramount that all process operating parameters (input parameters) that affect product quality attributes (output parameters) are established clearly during the PD phase of a new process, product, or a technology. This is accomplished typically by performing studies at lower and upper limits of the operating ranges generally referred to as the worst-case studies, crash studies or feed-stream studies. Some studies are performed up to the edge of failure and then stepped back to the ranges where process performance is acceptable. These studies can be simulated or performed with active ingredient or product derived from starting material generated during PD phase of the project. These studies can also be performed by generating starting material by artificially setting the parameters to the upper and lower limits of

the range. The process parameters are generally classified as critical process parameters and noncritical process parameters.

Critical Process Parameters

By definition the critical process parameters are "those operating parameters that directly influence the quality attributes of the product being produced." For example, temperature and pH in a fermenter are considered critical operating parameters as they have a direct influence on the viability of the organism and the chemical or biological activity of the product being produced. Other parameters that may be considered critical for fermentation processes are: cell viability, media conductivity, glucose concentration, oxygen and air uptake rates, and cell density in the production vessel or device.

Noncritical Process Parameters

The noncritical process parameters are "those operating parameters that have no direct influence on the quality attributes of the product being produced." For example, cell age and media flow rates in a fermenter are considered as noncritical operating parameters as they have no direct influence on the viability of the organism or the activity of the product being produced. Other parameters that may be considered noncritical for fermentation processes are: cell density in the inoculum, cell productivity, agitation rate, perfusion rate, and cell osmolality in the production vessel or device.

Cell Culture and Fermentation Process

A number of different approaches have been used to exploit cell culture technology and develop pharmaceutical products and medical devices, for example expression of the molecule of interest by cells through genetic manipulation or the use of cells as such for treating certain medical conditions. Of these, the technology based on product expression through genetic manipulation is most common. Commercial fermentation processes and bioreactor technologies have been developed in the last several decades to state of the art production of pharmaceutical agents of interest. The introduction of rEPO, rTPA, rFVIII, rInsulin, rHGH, rPDGF, etc. to treat many medical problems would have not been developed without these advances in the technologies. Figure 2 depicts a flow diagram for a typical fermentation process. We will discuss below the steps involved in the development of a commercial cell culture process in the light of process validation. Of special interest here is the establishment of critical and noncritical process parameters that will be verified during the process validation phase.

Cell Line Development

Once a clone has been selected for commercial development it is crucial that the nutritional requirements for the cell line must be defined. The cell line may need to be adapted for growth in certain cases, such as the expression and production of a product in a serum-enriched or serum-free media. The following nutritional requirements in terms of their concentration (% or molarity) or amounts (g/L or PPM or PPB as appropriate), and growth conditions must be established:

- Chemically defined growth medium
- Need for protein/serum/plasma or a protein-free media

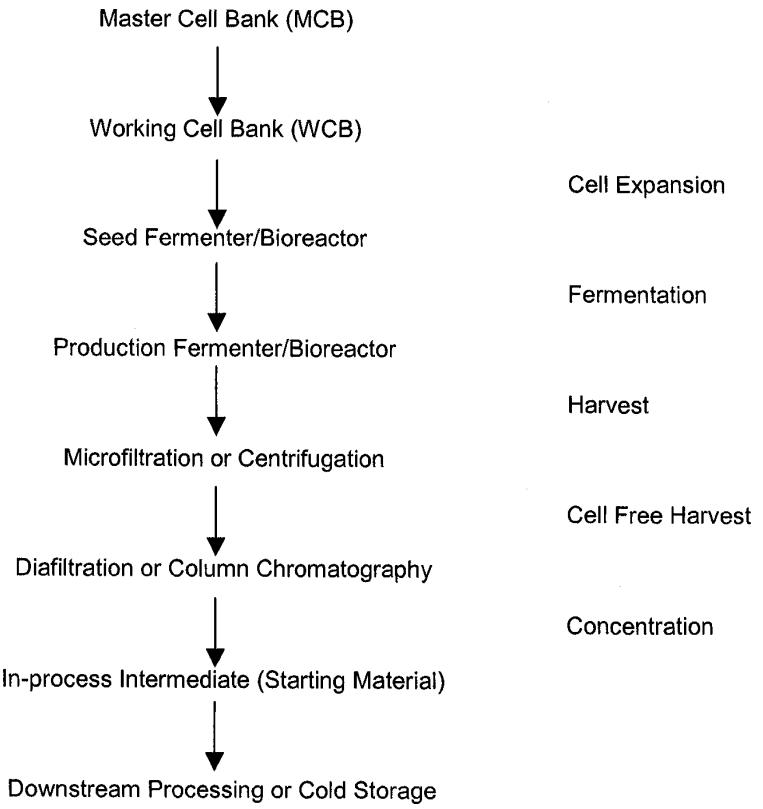

Figure 2 Typical fermentation process flow diagram.

- Requirements for vitamins or fatty acids
- Requirements of any special chemicals
- Requirements of growth factors or hormones, etc.
- Optimizatipon and maintenance of appropriate pH and ionic strength
- Requirements of oxygen, carbon dioxide or other gases
- Optimizatipon and maintenance of appropriate temperature
- Frequency of media changeover
- Frequency of harvesting of the cell line or product

Cell Line Characterization

The cell line must be fully characterized (17,18) for the absence of objectionable organisms or contaminants as follows:

- Absence of bacteria or spores
- Absence of fungi and mycoplasma
- Screening for adventitious viruses (nonretroviral)
- Screening for species-specific viruses (nonretroviral)
- Retrovirus contamination from other species

- Absence of other objectionable agents such as prions and TSE/BSE
- Phenotype/genotype characterization
- Identity and genetic stability

Master Cell Bank (MCB) Preparation

After selection of the appropriate clone for production purposes, the clonal cells should be expanded to appropriate fermentation scale, preferably to production scale fermentation, and used to prepare an MCB in appropriate sized vials for storage in liquid nitrogen. After preparation, the MCB should be scaled-up to evaluate its life cycle and productivity. The process parameters that need to be established at this stage are: cell concentration and viability for preparing MCB, cell volume in the vial, purity and amount of the preserving agent such as DMSO, storage temperature, and acceptable cell recovery and viability after thawing of the MCB vial.

Working Cell Bank (WCB) Preparation

The MCB is typically expanded up to seed or production scale fermentation to prepare the WCB in appropriate sized vials or bags suitable for storage at $-70^{\circ}C$ or colder. The WCB should also be scaled-up to production scale fermentation to evaluate its productivity and other growth conditions, and it should be fully characterized as outlined in ''Approach and Rationale'' to ascertain that it is free of objectionable organisms and contaminants. The process parameters that should be established at this stage are: cell concentration and viability for preparing WCB, cell volume in the vial or bag, purity and amount of the preserving agent such as DMSO, storage time and temperature, and acceptable cell recovery and viability after thawing of the WCB vial or bag.

Cell Expansion and Seed Preparation

Procedures (SOPs, BPRs, etc.) should be prepared that describe in detail all the steps for the expansion of cells starting from WCB through preparation of the seed for inoculation of the final-scale production device (fermenter, bioreactor, bag or bottle or vessel). The cell expansion procedure may require only a few steps or may have a number of steps before a seed is ready for inoculation of the production device. In addition, the seed may be used immediately to inoculate a production vessel or it may be stored further until use. Therefore, it is important to evaluate the process and identify the critical and noncritical process parameters for each process independently.

The examples of the process parameters that may be established at this stage are: thawing time and temperature, volume of media and size of flask or bottle for initial cell growth, time and temperature for initial cell growth, media pH, conductivity, temperature, glucose concentration, oxygen and air uptake rates, cell viability and cell recovery at different stages, cell density for scale-up to the final seed vessel (bottle or bag or fermenter or bioreactor), cell density and cell viability in the seed to be used for inoculation of the production device (bottle or bag or vessel or fermenter or bioreactor), and storage time and temperature for the seed (inoculum).

Production Scale Fermentation

Fermentation at the production scale may be carried out in a vessel (fermenter or bioreactor), bottle, or a bag depending on the product type. The product may be

the cells themselves, for which efficient cell growth may be critical, or the product may be a biochemical entity (enzyme, protein, hormone, etc.) expressed either in the cells intracellularly retained in the inclusion body or secreted out of the cell in the spent medium. In the latter case, the stability of the molecule in the spent medium should be explored as storage time and temperature for the harvest will be critical for the stability of the product. The process parameters required to be established for the fermentation are: cell viability and cell density in the inoculum, media pH, conductivity, temperature, glucose concentration, oxygen and air uptake rates, cell productivity, cell life span, agitation rate, perfusion rate, cell density, cell viability, and cell osmolality in the production vessel or device.

Continuous fermentation (perfusion). Continuous fermentation, where the product is generally secreted in the spent medium, is the most efficient and commonly employed technology for the production of biopharmaceuticals today. The main concept of this technology is to keep the cells alive as long as they produce a quality product. The number of days the cells are kept in a fermenter (fermenter days) varies depending on the cell type and established time period (weeks or months) for producing a quality product. In this approach the cells are expanded to desired optimum concentration and induced to adhere to coated (with proteins such as collagen) or noncoated acrylic beads where they can survive for many months as long as their nutritional needs are met. Fresh medium is introduced (perfused) and spent medium (harvest) is removed from the fermenter on a continuous basis. A number of cell sedimentation devices (conical, incline or plate settlers) are used to separate the cells from harvest. The cells are returned to the fermenter and the harvest is collected in a harvest tank or bag. The fermenters used in this technology are typically smaller in size (50–2500 L), as continuous perfusion of media allows sufficient volume of harvest collected on a daily basis.

Since the equipment used is more complex and the fermenter cycle is typically long (months), the validation effort is more rigorous for this technology. Establishment of acceptable fermenter days requires full cell characterization (see Cell Line Characterization) at the beginning (early), middle, and end (late) of fermentation to demonstrate that the cell characteristics do not change over time. In addition, product quality attributes are evaluated for the product derived from early, middle, and late stages of fermentation. These activities are performed and established during PD phase and confirmed during formal process validation (PPQ).

Batch fermentation. This nonperfusion technology is employed for products that are either secreted in the spent medium or expressed intracellularly. The cells or the harvest is collected for the isolation and purification of the product depending on the expression of the product in the cells or in the spent medium. The fermenter cycle is generally short (days) for batch fermentation process than for continuous fermentation process (weeks or months). This technology is most efficient for products that are expressed intracellularly where cell mass expansion is critical for productivity. It is less efficient for cell secreted products as the cost of operation is high. The fermenters used in this technology are typically larger in size (500–25,000 L), as it is a batch operation that allows collection of cells or harvest only once per fermenter cycle. Since this technology does not use cell sedimentation devices and fermenter cycle is short (days), the validation effort for batch fermentation process is less rigorous.

Cell mass expansion. This technology is similar to batch fermentation process except the main objective of the fermentation is to expand cell mass. It is used mainly for the products that are expressed intracellularly or where the cells themselves are used for medical treatment (as a medical device), and the cell mass is critical for

productivity. The fermenter cycle is short (days) for this technology than for continuous fermentation process (weeks or months). The fermenter sizes used in this technology vary depending on the requirements of the cell mass. Since the equipment used is simpler and the fermenter cycle is short (days), the validation effort for this fermentation process is less rigorous.

Though validation of fermentation process may be simpler for medical devices using biologically active cells, process validation for their formulation, storage, and delivery are more complex. Since mammalian cells are more fragile than protein molecules, their storage without impacting their quality attributes are more challenging. Demonstration of biological activity retention for a heterologus cell-based product during production, distribution, and storage is a daunting task. Cell characterization studies (see Cell Line Characterization) may have to be performed more rigorously after formulation, storage, and end of shelf life of these devices. In addition, the level of impurities and contaminants would also require rigorous investigation during these stages. Moreover, an assurance that the biological activity and safety do not impair and adverse reactions do not increase during these stages also needs to be demonstrated.

New approaches and future of cell-based therapies. The manipulation of cell culture technologies to generate unique therapies and medical treatments has just begun. Further development of these technologies would be essential for their impromptu use in new ways to treat diseases. Mammalian cells are being evaluated for grafting, transplantation, tissue regeneration, and organ culture. Stem cells are being developed into numerous cell-based treatments/cure for many diseases such as cancer, HIV, Alzheimer's, Parkinson's, etc. Gene therapy is expected to be the ultimate cure for many diseases in the 21st. century. This field is expected to grow exponentially in the next 25 years and bring numerous challenges for cell culture scientists. A number of microbial hosts (bacteria, plasmids, viruses, etc.) are being evaluated as carriers or vehicle for gene therapy products. For a successful gene therapy product it is crucial that it is free of any side effects, is long lasting, and is fully effective. To accomplish these goals the gene therapy products would have to be pure, free from undesirable components, easy to use, effectively targeted to desirable site, effective transformation and expression of desired genes, complete correction or deletion of defective genes, and they must prove to increase longevity. Cell culture scientists would definitely address all these issues and develop appropriate technologies to attain desired results. However, imagination for validation of all these diverse technologies and processes is mind-boggling today. New approaches to validate these technologies, production equipment, and production processes would have to be developed to meet yet to be established regulatory requirements.

Product Isolation, Separation, and Concentration

The procedures for the isolation of the product, its separation from impurities and contaminants, and its concentration by different technologies, depending on the product type, are performed after the fermentation process is complete. The cell separation techniques such as centrifugation or microfiltration may be applied to concentrate cells for product recovery or remove cells from the harvest that contains the product. The parameters that may be critical for process validation for this process are: cell concentration and viability in the fermenter effluent (spent media with or without cells), storage time and temperature for the effluent, centrifugation speed or microfiltration rate for cell separation, cell separation time and temperature, and

storage time and temperature for the concentrated in-process intermediate (IPI, starting material).

In-Process Intermediate (IPI) or Product Preparation and Stabilization

The IPI may be the cell suspension or cell extract or concentrated harvest fluid depending on the product type. Some of the processes require stabilization of the IPI for storage prior to further processing of the product. In these cases a formulating agent may be added to the concentrated IPI prior to its storage in the cold. The parameters that should be considered for process validation here are: the purity and concentration of formulating agent, mixing of the formulating agent with the IPI, freezing time and temperature for the formulated IPI, and storage time and temperature for the formulated IPI.

For medical device applications, the IPI (cell suspension/tissue) may be the final product that may require cleaning and removal of impurities, formulation for stabilization, and preparation of the product for clinical use. In such cases, the parameters that need to be established for process validation are: amount of contaminants (DNA, proteins, etc.) in the final product, cell/tissue morphology, genetic characterization of the cells/tissue, amount of formulating agent, storage time and temperature, and shelf life of the product.

Downstream Process Development

The pharmaceutical or therapeutic proteins expressed in cells are further purified from the IPI generated during the fermentation. The purification steps may involve microfiltration/diafiltration, salt or solvent fractionation, and column chromatography (ion exchange, hydrophobic interaction, affinity, gel filtration, etc.). The examples of critical process parameters that should be established here are: pH, conductivity, salt or solvent residues, membrane life cycle, column operation parameters (equilibration, load, wash, elution, regeneration, storage, cleaning, life-cycle, etc.), impurities and contaminant clearance (DNA, microbes, viruses, proteins, etc.), and hold times and temperature for in-process material and equipment. There is a whole battery of process parameters that should be established for process validation for these operations. It is beyond the scope of this book chapter to go into details of these parameters, but they are discussed in more detail elsewhere (19).

Bulk Formulation, Stabilization, and Final Packaging

The purified in-process material (bulk) is typically formulated for stabilization and either stored until further processing or filled and/or freeze-dried depending on the mode of application for the product. These operations are generally performed in the clean room environments (class 100) that require detailed and cumbersome PQ studies. At this stage, the product is characterized in detail and every lot of the product is evaluated against pre-established specifications and quality attributes. These include: product amount (units or weight), purity, strength, pH, ionic strength, amount of trace metals, amount of impurities and contaminants (DNA, proteins, microbial load, endotoxins, etc.), amount of residual reagents (solvents, polymers, chemicals, etc.), amount of formulating agents (excipients, sugars, etc.), sterility, storage time and temperature, and product shelf life. The process parameters governing these quality attributes should be considered as critical

parameters during process validation studies. The filled or lyophilized product vials are inspected for container closure integrity and labeled appropriately. The product coming from validation runs is placed on stability to demonstrate that the product is stable during its entire shelf life. In addition, shipping studies are performed to demonstrate that the packaging is shatter-proof and the product is stable during shipping. All appropriate process parameters covering these quality aspects of the process should be established and verified during the process validation studies.

Process Validation

Process validation projects are complex and cover a great deal of details. The critical steps for performing process validation studies are depicted in Fig. 3. The process validation begins with the receipt of a PD report from the R&D arm of an organization, and ends with the approval of a process validation report and applicable SOPs/BPRs by the QA arm of the organization. A well-written PD report with adequate details of the developed process greatly facilitates process validation. In addition, efficiency and success of a process validation depends heavily on the extent and quality of PD work and/or engineering runs performed on associated equipment (see Process Validation Protocols and Final Reports for details). Prior to formal process validation, processes should be run rigorously on associated equipment to gain valuable experience. The experience gained is well worth as it saves time and resources during the formal validation stage by efficiently resolving any unforeseen problems, discrepancies or deviations. In no event, the engineering runs or PD studies be left for performance during the formal process validation stage (PPQ phase). Successful completion of a process validation study requires good coordination among the responsible departments such as production/manufacturing, quality control, QA, engineering, R&D, regulatory affairs, etc. We will discuss below the types of processes that are generally covered under process validation projects (PV or PPQ).

- Manufacturing procedures for fermentation, product isolation, purification, formulation, sterilization, filling, and freeze drying of products
- Microfiltration, ultrafiltration, and sterile filtration
- Cleaning procedures for equipment and processes (clean-in-place, CIP)
- Lifecycle determinations for chromatographic resins, membranes and filters
- Impurity/contaminant clearance (DNA, viruses, host cell proteins, etc.) studies
- Impurity/contaminant inactivation (virus, endotoxins, TSE/BSE, etc.) studies
- Sterilization and steam-in-place (SIP) systems
- Critical utilities such as water for injection (WFI). (PPQ is performed to demonstrate process reproducibility/consistency and product quality)
- Environmental qualification for facilities (EQ). (EQ is a combination of air handling, equipment/facility cleaning, sanitation, gowning, and environmental monitoring)
- Re-processing of process intermediates, bulk, and final product
- Revalidation of processes due to major change control requests (CCRs)

Unlike equipment re-qualification, revalidation of a production process is currently not a mandatory cGMP requirement, but must be considered after implementation of a large number of CCRs or as soon as a process shift is noticed. The

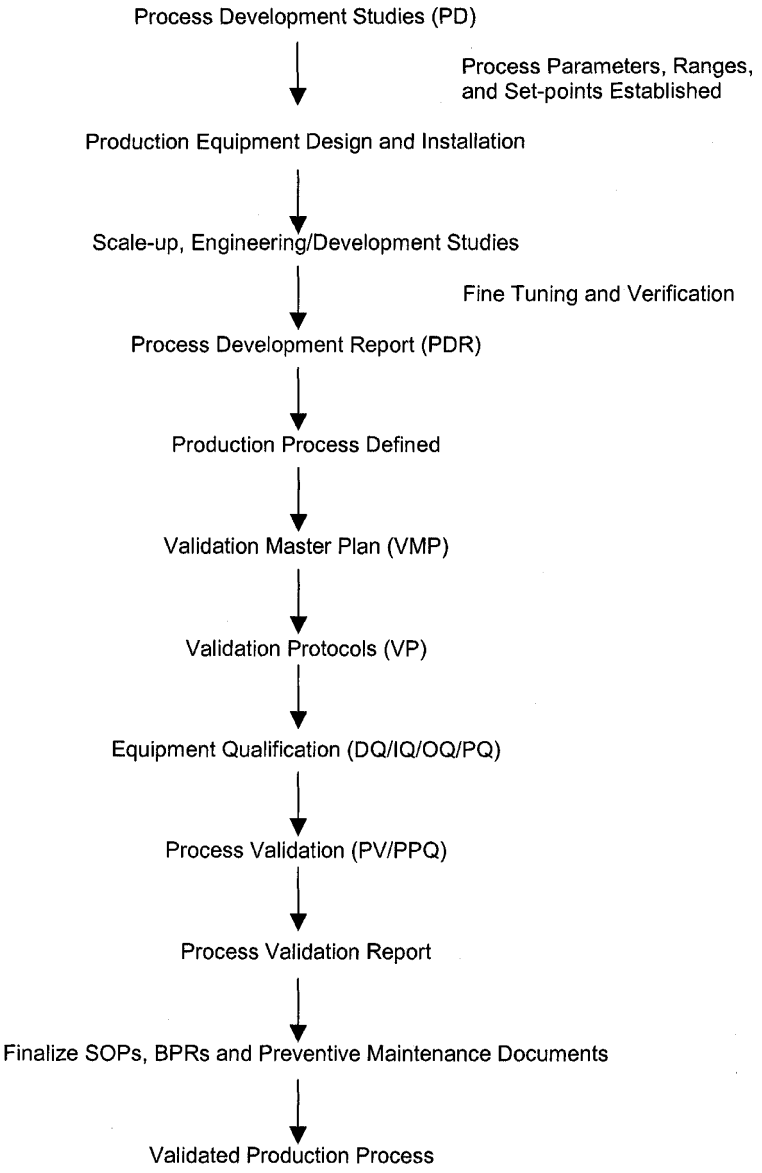

Figure 3 Critical steps leading to process validation.

purpose of the process revalidation should still be to demonstrate that the implementation of CCRs or an observed shift in the process has not affected quality attributes of the final product. Even if no major changes were implemented or process has not shifted, process revalidation should still be considered to demonstrate that the process is running within controlled limits, at some appropriate time intervals after original process validation (after 100/ 500/1000 lots or 2/5/10 years, as appropriate).

Life Cycle of Process Validation

Process validation is a continuous process, it does not end after the sign-off of the original process validation report. Figure 4 illustrates the life cycle of process validation by keeping the process and product quality attributes in mind. It is not uncommon for companies to initiate change control requests within days or weeks after the completion of the original process validation. Many companies have made major process changes within one year of the original process validation.

Since cell culture and fermentation processes are continually evolving, changes are being made to manufacturing processes on a regular basis by many companies. In fact, a number of second-generation products are derived from the same cell lines, except the cell lines have been adapted to produce the product in a protein-free (serum/plasma) media.

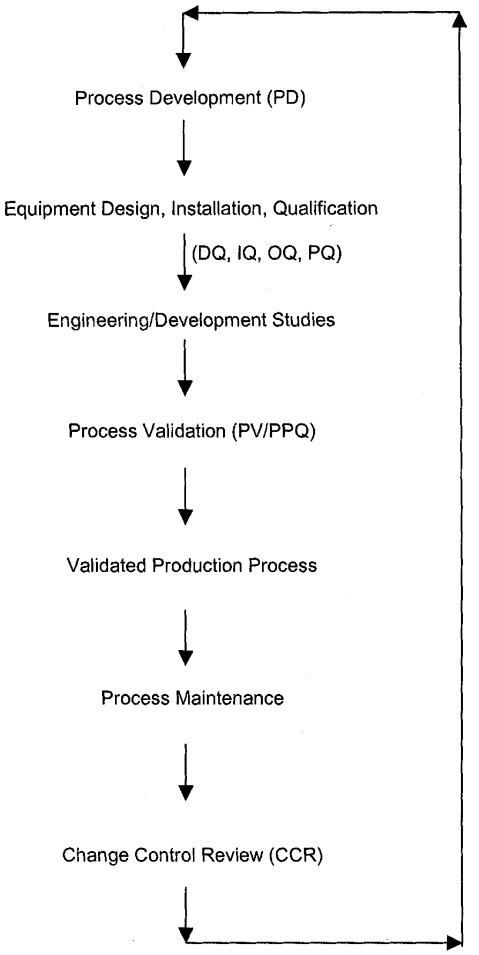

Figure 4 Life cycle of process validation.

Though such changes improve the product quality and safety tremendously, the process validation task is nevertheless the same. Such changes often lead to the construction of a new manufacturing plant and complete re-validation of the production process. Therefore, the life cycle of a manufacturing process after original validation is mainly dependent on the volume of change control activity.

Though regulatory agencies have not made a guideline yet for process re-validations, they have been advising drug/biological manufacturers for some time to evaluate the need for process re-validation at some regular intervals. Many companies do not feel the need for process re-validations based on the activities that are covered under their change control management programs. However, it may be prudent to perform process re-validation after a major process change or after a number of small changes to assess the cumulative effect of many change control requests. One possible mechanism may be to perform process re-validations after every 100/200/500/1000 production runs or, 2/5/10 years or sooner, as justified by the evaluation of the historical process input and output parameters, specifications, set-points, action limits, or other observations that suggest that the validated process may have shifted.

VALIDATION OF THE MANUFACTURING PROCESSES AND ASSOCIATED EQUIPMENT QUALIFICATION

Validation SOPs

Establishment of sound validation SOPs and strict adherence to them is crucial for the success of any validation project in a cell culture-derived drug/biological manufacturing organization. These SOPs are typically written by validation professionals and are approved by the responsible groups (e.g., operations, engineering, validation, research, PD, QC, QA, and regulatory affairs) that have a stake in the validation projects. The SOPs should be established for the required validation functions, as listed below:

- Site or facility validation policy and management
- Validation master plan and final report
- Validation requirements for DQ, IQ, OQ, PQ, PD, PPQ, EQ, and RQ validation protocols
- Design of worst-case studies for OQ, PD, and PPQ protocols
- Determination of acceptance criteria for validation protocols
- Design of prospective or concurrent validation studies, and retrospective data analysis
- Write a validation protocol and a validation final report
- Revision of a validation plan, protocol or a final report
- Execution of IQ and OQ protocols
- Execution of engineering and PD protocols (studies)
- Execution of PV or PPQ protocols (studies)
- Validation of analytical methods, assays, and procedures
- Performance of validation studies on clearance and/or inactivation of impurities contaminants
- Performance of filter qualifications and sterile filtration validations
- Performance of cleaning validation studies on equipment, accessories, and processes

- Performance of sanitation and sterilization validation studies
- Performance of validation studies on automated systems (computers, DCS, and PLCs)
- Performance of environmental qualification (EQ) studies
- Review, verification and analysis of validation data and documents
- Documentation of validation discrepancies and deviations
- Performance of re-qualification (RQ) studies on validated equipment, systems and processes
- Establishment of a validated lifecycle (for cells, resins, filters, membranes, etc.)
- Requirements for maintaining a equipment or a process in a validated state
- Display of validation status (labels) for validated equipment and systems
- Training and certification program for personnel involved in validation projects

In addition to these, many other SOPs may be established for performing specific validation functions depending on the need of the equipment, system or process (e.g., determination of equipment surface finish, calibration of instruments and thermocouples, determination of agitation rates, determination of HETP and Af on chromatography columns). The required SOPs are typically identified and established by qualified validation professionals depending on the need of an organization.

Validation Master Plan

It is paramount that a detailed VMP should be written before implementation of any new or unlicensed process for the production of a cell culture derived drug/biological product (11,12,20–22). The VMP provides details of an organization's plans for carrying out all validation activities on production equipment, systems, and processes. The plan should provide details of equipment, facilities, utilities, raw materials, storage times and temperatures, environmental requirements, production processes, critical and noncritical process parameters, process set points, SOPs and BPRs, in-process and final product sampling, analytical assays and methods, QC testing, in-process testing and acceptance criteria, and product release specifications. The VMPs should be approved by all responsible stakeholders or senior management of a company (operations/manufacturing, QA, QC, engineering, PD, and regulatory affairs), and should contain the following information at a minimum:

- Objective
- Scope and rationale
- Process and product design
- Description of manufacturing facility
- Equipment description and qualification (DQ/IQ/OQ/PQ)
- Process description and qualification (PQ/PPQ)
- Description of utilities and supplies
- Description of automated systems
- Equipment cleaning (CIP/COP) and sanitation (SIP)
- HVAC and EQ
- Process parameters and set points
- In-process testing and product specifications
- Analytical methods and procedures
- Manufacturing procedures and batch records

- Responsibilities
- Execution plan and schedule
- Documentation and training
- Modification and change control
- Preventive maintenance
- References
- Attachments
- Facility diagram
- Process flow diagram
- List of validation protocols
- List of PDRs
- List of engineering reports
- Project schedule and Gantt charts
- Other pertinent documents

Equipment Qualification

A great deal of information is available in the literature, web sites of the regulatory agencies (FDA, EMEA, ICH), professional societies (PDA, ISPE, AAPS, etc.), various seminars and symposia, and from professional consultants for the qualification of process equipment. The key components of equipment qualifications are covered in the following validation studies:

- Design qualification (DQ)
- Installation qualification (IQ)
- Operational qualification (OQ)
- Performance qualification (PQ)

Equipment qualification studies should be performed for all process equipment (fermenters/bioreactors, chromatography, microfiltration/ ultrafiltration systems, tanks and vessels, autoclaves, CIP and SIP systems, freeze dryers, etc.), utility equipment (WFI, clean steam, solvent delivery, etc.), supply equipment and accesories (gases, filters, raw materials, etc.), automated systems (computers, DCS, PLCs, etc.), and critical facility equipment (HVAC, warehouse, storage chambers, shipping containers, etc.). It is beyond the scope of this chapter to cover details of equipment qualifications. The readers can review selected references (11,12,22,23) to learn more about equipment qualifications.

Equipment Engineering Runs and Process Development Studies

After completion of the DQ, IQ, OQ and PQ studies on the equipment, it is prudent to perform equipment engineering runs and PD studies (pre process validation studies, also known as trial runs) to ascertain that the developed process is scalable to the production scale. These studies confirm that the process can perform effectively within the ranges established at the small-scale or previously developed scale. These studies also help in ensuring that the formal process validation studies would not run into major discrepancies, deviations, or failures. They also provide an opportunity to fix any problems that may have been ignored previously. Furthermore, they provide a chance to develop or fine tune acceptance criteria and specifications for the formal process validation studies down the road.

These studies are performed under approved PD protocols (see Cell Culture and Fermentation Process). Typically three runs are performed at the production

scale by following the SOPs and BPRs written for the process. All sampling and testing is performed as in the process validation studies except that some tests may not be required (have no chance of failure due to scale of operation). PD final reports are prepared after review and analysis of all results and associated data. Appropriate conclusions are drawn and recommendations for formal process validation studies are made in the final reports. The examples of these studies are equipment load studies (autoclaves, depyrogenation ovens, viral inactivation tanks, pasteurization, column chromatography, cleaning validations, etc.), mock runs (process runs without active ingredient), partial load studies (part load with active ingredient and remaining load with an excipient), and full process runs with active ingredient. In conclusion, these studies provide peace of mind that the formal process validation would be un-eventful or would be completed with minimum difficulties.

Process Validation Protocols (PV or PPQ) and Final Reports

Process validation protocols are written to demonstrate that the production processes are reproducible, are in control, and consistently produce a product of predefined specifications and quality attributes. The protocols are also used to demonstrate impurity/contaminant clearance, validation of operating ranges, equipment cleaning, and establishment of lifecycles for chromatographic resins, filters, and membranes. It is indisputable that well written process validation protocols are instrumental for efficiency and success of process validation studies. The protocols should contain details of the production process and equipment to be used, SOPs and BPRs to be used, critical and noncritical process parameters, process set-points and action limits, sampling and QC testing, analytical methods and assays to be used, and in-process and final product specifications. The process validation protocols should be designed to incorporate worst-case studies based on the acceptable level of risk for the process and the product. It is important that all pre-requisites (e.g., DQ/IQ/OQ/PQ on equipment or a system, approved SOPs/BPRs, instrument calibration, and personnel training) are completed before execution of a process validation protocol.

After execution of the validation protocols and completion of all testing, validation final reports should be written with complete details of protocol execution (BPRs and sample tables), test results, discrepancies and deviations, modifications or change control requests, passage/failures against acceptance criteria, statistical data analysis, preventive measures and maintenance, validated critical and noncritical process parameters, process set-points and action/alert limits, supporting data, and conclusions. The validation protocols (and final reports after completion of the study, see below) should be reviewed and/or approved by all responsible parties such as manufacturing, PD, engineering, quality control, QA, validation, and regulatory affairs (optional), as appropriate, prior to its execution. A typical outline of a process validation protocol and final report (validation package) are given below:

- Approval signatures page
- Objective
- Scope and rationale
- Process description
- Acceptance criteria
- Responsibilities
- References

- Prerequisites
- Validation procedures or test functions
- Result, data analysis and discussion
- Validated critical and noncritical process parameters
- List of SOPs and BPRs
- List of supporting data and documentation
- List of discrepancies and deviations
- Conclusion

The validation final reports must contain all results obtained during the execution of a process validation protocol. All deviations should be described along with justifications for their acceptance. The results must be evaluated against the pre-established acceptance criteria, product specifications, and quality attributes. The data should also be evaluated statistically and confidence intervals for the data should be calculated to demonstrate process robustness. Process capability (Cpk) calculations should also be performed to demonstrate process reproducibility. The final report should draw a scientifically sound conclusion based on the results obtained during the study. Ranges and set-points for all validated critical and non-critical process parameters should be established. In addition, alert and action limits for validated process parameters should also be established wherever applicable. Moreover, a plan to monitor process parameters during production should be devised, and the historical data should be evaluated statistically at pre-established time periods (yearly or after every 100 lots or other suitable interval). Process capability (CpK) calculations should also be repeated on historical data to demonstrate process reproducibility. The in-process action and alert limits and product specifications should be re-evaluated and tightened, wherever possible, after complete data analysis at pre-established time periods.

Raw Materials

The quality of raw materials plays a major role in attaining quality attributes for the final product and indirectly affects success or failure of process validation. The critical raw materials for a cell-derived product during fermentation are: basal media, purified water or WFI, salts and buffering agents, oxygen and carbon dioxide, amino acids, vitamins, glucose, serum or plasma proteins (animal or human), and other nutrients such as hormones or growth factors. It is essential to establish specifications for all raw materials used in the production process, and ensure that quality attributes of all incoming raw material lots are met against the established specifications. Any changes made to the specifications for raw materials should be evaluated through change control management and process validation(s) performed wherever necessary. In addition, FIFO (first in first out) procedures should be established for approved/released incoming raw material lots.

Facilities

All equipment installed in a facility must be qualified (DQ, IQ, OQ and PQ, wherever applicable). The typical facility related equipment includes HVAC, cold/freezer freezer rooms, cooling towers and heat exchangers, chemical/solvent tanks and distribution system, and waste treatment and disposal systems. The facility cleaning procedures are established based on the requirements for each classification (class

100, 1000, 10,000, and 100,000; or grade A, B, C, or D), and must be validated under the facility EQ. Emergency power systems should also be qualified (DQ, IQ, and OQ) to demonstrate that uninterrupted power supply is available to critical production equipment (cold rooms, freezers, freeze dryers, etc.).

Utilities

The critical utility systems must be qualified (DQ, IQ, OQ, and PQ). The examples of utilities that are used in a typical cell culture based production facility are potable water, purified water, WFI, plant steam and clean steam systems, gas distribution systems, CIP and SIP systems, and electricity supply systems.

Production Equipment

Besides equipment qualification (DQ, IQ, OQ, and PQ), typical production equipment used in a cell culture-based facility must be validated (PPQ). The production equipment includes media and buffer preparation equipment, media filtration equipment, media storage tanks, media and buffer distribution system, seed fermenters or bioreactors, production-scale fermenters or bioreactors, aseptic transfer equipment, cell settlers, heat exchangers, pumps and agitation systems, gas sparging equipment, harvest tanks, microfiltration and ultrafiltration systems, centrifuges, and initial capture and concentration equipment. These validation studies may be performed separately or may be combined in the process validation study for a new process, product or technology.

Instruments

Though all instruments associated with equipment systems are generally covered in the equipment qualification, any stand-alone instrument must be qualified separately. In addition, all instruments must be calibrated at the time of their qualification and must remain on a regular calibration schedule after their qualification. The established calibration schedule must be justified and scientifically sound. All test instruments used to measure a specific parameter during validation must also be calibrated.

Analytical Equipment, Instruments, and Methods

All analytical equipment and instruments must be qualified (DQ, IQ, OQ, and PQ), and associated methods, procedures or assays must be validated (PPQ). During this phase of validation, the procedures must be evaluated for precision, accuracy, repeatability, and variability. The results must be evaluated statistically to demonstrate confidence intervals for each sampling condition. The instrument to instrument, operator to operator, and intra- or inter-assay variabilities must be established.

Distributed Control Systems (DCS) and Programmable Logic Controllers (PLCs)

All automated systems (computers, DCS and PLCs) must be qualified (DQ, IQ, OQ, and PQ), and associated software must be tested for its intended function, use, and

its lifecycle (24–26). The validation aspects of these systems are verified and confirmed during the process validation phase of the validation activity.

Cleaning and Disinfecting

The cleaning of the equipment including the CIP systems must be qualified (DQ, IQ, OQ, and PQ), and the procedures must be validated (PD, PV, and PPQ). The cleaning validation studies must demonstrate that the process residues and cleaning agent residues are removed to the acceptable levels after the cleaning (27). The acceptable levels for the residues must be established by actual scientific data or sound scientific knowledge (28–31). Appropriately validated assays to test the residues must be used. In addition, all cleaning and disinfecting agents must be qualified or approved for use. The cleaning requirements for each step of the process must be established per appropriate guidelines established by regulatory agencies (27–29). Since the final rinse in cleaning processes for biological products is performed by WFI, many companies use the quality attributes of WFI as acceptance criteria for rinse samples. In addition to rinse sampling, cleaning agent residues and process residues (protein, fatty acids, nucleic acids, raw material components, etc.) are tested by surface swabbing and evaluated against pre-established acceptance criteria. The typical assays used for cleaning validations are pH, conductivity, TOC, microbial load, endotoxin, protein assays/analysis, and other assays for specific residues. A cleaning monitoring program should be established to maintain the equipment in a validated state (31).

Standard Operating Procedures (SOPs) and Batch Production Records (BPRs)

The appropriate SOPs and BPRs must be drafted, reviewed, and approved prior to beginning of process validation studies. These procedures may be modified, if needed, with appropriate justifications during validation as long as the last validation runs (three or more) are performed after making the modifications and all validation acceptance criteria are met. The modifications to the procedures during validation should be made through the change control system of the organization. It is important that all changes made during validation are fully incorporated in the SOPs and BPRs prior to approval of the final validation package.

Personnel and Training

The success of a process validation project depends solely on two things—first an effective and detailed technology transfer program, and second an effective and detailed training program for the new processes or technologies. Therefore, it is crucial that the organizations establish a very effective and practical technology transfer function, and an effective and practical training program for the operations personnel. The initial training is typically provided by the R&D arm of the organization (or whoever developed the process and has the most knowledge or experience). The training program and procedures must be well documented and must follow cGMP guidelines.

Documentation

The regulatory agencies have stated in no uncertain terms that the lack of documentation (even if the work was performed) would be interpreted as if no work was

performed. Therefore, the importance of documentation cannot be emphasized enough. The following documents need to be in place for the purposes of process validation:

- An approved validation master plan/report, if applicable.
- An approved validation protocol/report/final package.
- Original or copies of all approved specifications or acceptance criteria.
- Original or copies of all PD reports.
- Original or copies of all approved process validation parameter documents.
- Original copies of all prerequisite sheets completed during validation.
- Original copies of all validation attachments/execution documents.
- Original or copies of all SOPs/BPRs employed during validation execution.
- Original or copies of all QC test reports.
- Original or copies of all other test reports.
- Original or copies of all raw data, or location of all archived raw data.
- Original or copies of all supporting data related to validation.
- Original or copies of all change control requests implemented during validation.
- Original or copies of all deviation reports encountered during validation.
- Original or copies of all corrective action reports, if applicable.
- Original or copies of all SOPs/BPRs/documents revised during or as a result of validation.
- Original or copies of all other documents related to validation.

Preventive Maintenance

Maintenance of validation post-licensure is as important as original validation, as it transforms into cGMP compliance after licensure of the production process. To maintain a process in the validated state, it is crucial that procedures for preventive maintenance (PM) be established prior to original validation, modified as appropriate during validation, and are followed thoroughly and timely after validation. In addition, the processes should be monitored regularly with respect to process parameters observed during production. The historical process parameters data should be evaluated at some fixed intervals (annually or sooner if needed) with respect to validated parameters and be tightened or loosened (in principle) as appropriate and justified by the data based on sound scientific principles and policies. The historical data must be evaluated by applying appropriate statistical methods and calculation for process capability (Cpk). The procedures for the following activities should be established as needed:

- Evaluation of production equipment, parts, and accessories.
- Calibration program for all instruments used in production and testing.
- Equipment re-qualification and process re-validation program.
- Preventive maintenance program for equipment, facilities, and processes.
- Change control program.
- Evaluation of validated process parameters.

Change Control

A change control system must be instituted to document all changes made to validated production processes (32). The change control activity begins after the installation of the equipment systems and continues throughout the lifecycle of the process

or product. The change control requests (CCRs) should be initiated prior to making the change, except in emergency situations, and must be reviewed and approved by all involved groups. The impact on validated systems and processes must be assessed and any identified validation work must be completed before closing the CCR. If the change is minor, little or no validation work may be required, however if the change is major, full revalidation may be required. The US FDA published a guideline in July 1997 (33) that requires notification of all changes to the agency depending on the extent of the change in the following manner:

Major changes require submission and approval of a supplement prior to the distribution of the affected product. *Moderate* changes require submission of a supplement at least 30 days (CBE30) prior to the distribution of the affected product. *Minor* changes do not require any submission prior to the distribution of the affected product, but must be documented in the annual report.

MANUFACTURING PLANT QUALIFICATION

The manufacturing plant qualification and licensing requires additional validation, testing, and documentation besides equipment qualification, utilities/facilities qualification, process validation, and establishment of PM and change control programs. Successful completion of the following items is key to the licensure of a manufacturing plant for a cell culture-derived pharmaceutical or therapeutic or diagnostic product:

Plant Design and Construction

The manufacturing plant must be designed and constructed per appropriate local, state and federal regulations and bylaws. The plant must be built by keeping the product and personnel flow in mind. In general, the product flow should be unidirectional. All required essential utilities (power, water, waste disposal, sewer, etc.) and facilities (warehouse, receiving, shipping, etc.) must be planned for and built into the manufacturing plant. Location and installation of all production equipment, process utilities, facilities, and support systems must be designed and procured per requirement of the production process. Additional details for manufacturing plant design and construction are available elsewhere (8,20,21).

Validation Master Plan

A VMP must be written, approved by the management, executed, and revised/updated as needed starting with the site selection and plant construction and ending with the licensure of the manufacturing plant (see Process Development Report Section for the details that must be included in the VMP).

Equipment Installation

The production equipment, utilities/facilities equipment, and all support system equipment as designed for the process must be procured from quality manufacturers and installed per manufacturer's recommendations in the manufacturing plant. All essential utilities (power, water, disposal, etc.) and supplies (gases, solvents, etc.) must be available prior to the installation of equipment systems. Equipment check-

outs (ECOs) must be performed to evaluate safe and normal operation of the equipment prior to equipment qualification. It is also essential that the equipment life cycle be established at this phase to ensure that the equipment performs as designed during the entire life of the equipment.

Equipment Qualification

Please refer to Equipment Engineering runs and Process Development Studies, and Process Validation Protocols and Final Reports Sections for details on equipment qualification. It is crucial that ECOs and dry runs (trial/engineering runs) are performed during the equipment qualification phase to ensure that the equipment operates within the ranges as designed for the process and certified by the manufacturer.

Equipment Performance Qualification

These studies are performed during the qualification phase of the equipment to ensure that the equipment delivers the desired output for the manufacturing process such as flow rates, temperature, pH, conductivity, agitation rates, sparging rates, cell retention, bioburden reduction, endotoxin removal, depyrogenation, sterile filtration, sterilization, etc. Equipment loading studies (autoclave loads, vial washer loads, depyrogenation loads, stopper processing loads, chemical inactivation tank loads, etc.) are performed during this phase to ensure that the equipment loads are processed appropriately to meet all quality attributes desired in the qualified loads. It is essential to evaluate which of the equipment needs to be placed on equipment re-qualification program to ensure that equipment delivers desired quality attributes in the processed loads during the entire life of the equipment.

Heat, Ventilation, and Air Conditioning (HVAC)

The qualification of HVAC system (DQ, IQ, OQ, and PQ) for the manufacturing plant must be completed during the validation phase. This includes qualification of the air handlers, HEPA filters, distribution piping, and associated equipment. The PQ on the HVAC system must demonstrate that the adequate air flow and particle levels (viable and nonviable) are achieved per specification (Federal Standard 209E or European ISO Standard or equivalent) for the different manufacturing environments (class 100, 1000, 10,000, and 100,000; or grade A, B, C, D by European standards).

Cleaning and Sanitation

Effective equipment and facility cleaning and sanitation procedures play a major role in maintaining equipment in a validated state and maintaining its life cycle, and by doing so, assures maintenance of product quality attributes (27–31). Cleaning and sanitation procedures should be developed that can effectively clean equipment product contact surfaces, working and processing areas, work surfaces, floor and wall surfaces, and drainage and disposal systems. The cleaning/sanitation procedures must be validated by using appropriate acceptance criteria. The specifications or acceptance criteria for cleaning and sanitation should be established on a case by case basis by consideration of the manufacturing process, processing time, and

in-process materials used. Hold times for process equipment after cleaning must be established and demonstrated (qualified). PD studies should be performed to evaluate effectiveness of the cleaning agents and procedures and establishment of acceptance criteria before PPQ studies are performed. The typical tests used to demonstrate cleaning of equipment product contact surfaces are visual examination, microbial load, endotoxin level, pH, conductivity, level of residual process impurities, level of residual cleaning agents, TOC, and any other appropriate test for a residue. The typical tests used to demonstrate effective sanitation procedures are visual examination, levels of viable particles, level of nonviable particles, and absence of objectionable organisms. The following cleaning and sanitation procedures should be established at a minimum:

- Procedures for cleaning inner and outer surfaces of equipment.
- Procedures for cleaning of chromatography columns, filters and membranes
- Recipes and methods for CIP/COP systems.
- Manual cleaning procedures.
- Cleaning/sanitation procedures for work surfaces, floors, and walls.
- Cleaning/sanitation procedures for drains and disposal systems.
- Cleaning/sanitation procedures for utilities (WFI, gases, steam, etc.)

Gowning and Personal Safety

Appropriate and protective gowning is essential for preventing the product from getting contaminated by human interaction and also to safeguard humans from any undesirable effects due to exposure of finished products, in-process materials, process chemicals, and supplies or accessories used during production. The personal safety devices used are lab coats or gowns, eye/hearing protection devices, gloves, face shields, and chemical or solvent handling devices. Clear procedures should be written for effective use of gowning and personal safety devices.

Environmental Qualification

In addition to qualification of HVAC, equipment/facility cleaning, and gowning; an EQ should be performed to demonstrate clean manufacturing environment as a combination of an effective HVAC system, effective equipment cleaning procedures, effective gowning procedures, and effective facility cleaning procedures at dynamic and at rest conditions. The acceptance criteria for the EQ for class 100,000, 10,000, 1000, and 100 (grade A, B, C, and D by European Standard) are different and are derived from Federal Standard 209E and other US and European regulations. The typical documentation and testing performed during EQ are as follows:

- Number and gowning status of the operators present during dynamic conditions.
- Number and gowning status of the operators present during rest conditions.
- Swab testing of operator's gowns for presence of viable and nonviable particles.
- Verification of air flow changes/hour and air flow rates during testing.
- Testing of viable and nonviable particles in the air. Testing of viable and nonviable particles in swab samples from work surfaces, floor surfaces, and walls.
- Verification of all cleaning performed during the testing period.

- Presence/absence of objectionable organisms in the environment.
- Discrepancy investigations and implementation of corrective actions.
- Establishment of an environmental monitoring program.

Preventive procedures to check the presence of insects, pests, reptiles, and rodents should also be employed.

Capacity Evaluation

Though not a regulatory requirement yet, regulatory agencies sometimes request that a production capacity evaluation be performed for each manufacturing facility that requires licensure. Capacity evaluation should be performed by verifying the capacity of the equipment, utilities, supplies, facilities, raw materials, in-process materials, and final product production. The following items should be evaluated for the capacity assessment of the manufacturing facility:

- Capacity for manufacturing areas in terms of space and production for equipment such as seed and production fermenters, media/buffer tanks, harvest tanks, filtration/microfiltration, ultrafiltration/diafiltration, chromatography columns and skids, in-process material and bulk storage tanks, filling machines, lyophilizers, vial washers and processors, stopper washer and processors, cold rooms and freezer rooms, refrigerators and freezers, warehouse capacity, storage of raw materials and process intermediates, and storage of quarantine and released product.
- Capacity for processing/storage of potable water, purified water, WFI, plant steam, clean steam, CIP, SIP, cooling towers, heat exchangers, gases, etc.
- Capacity or production/storage of critical raw materials such as plasma, serum, hormones, peptides or proteins, affinity-matrix for columns, etc. used in the production.
- Capacity in terms of processing supplies such as autoclave loads, depyrogenation oven loads, vial/stopper processor loads, filters, etc.
- Capacity in terms of cleaning of equipment and turnaround time.
- Capacity for maintaining appropriate manufacturing environment
- Capacity in terms of personnel for working space, production schedule training, document archival, etc. performed in an orderly and a normal way.

A capacity report should be prepared and approved by responsible departments after evaluation of the above listed and any other requirements for capacity. The capacity report should clearly demonstrate the plant capacity in terms of product produced per day/week/month/year based on the capacity of each equipment, process, schedules, and trained personnel.

Access and Security

The manufacturing areas should be accessible only to qualified and authorized personnel. Adequate security procedures should be established to demonstrate that the manufacturing facility is secure and not accessible to unauthorized people. Regulatory agencies have been paying special attention to this issue lately due to recent product counterfeiting incidents in generic drug companies (34).

Building Licensure

A BLA is submitted to the FDA or its equivalent EMEA or other global regulatory agencies, after completion of the above listed and other required activities and documentation, for licensure of the manufacturing facility. A pre-approval inspection (PAI) is often performed by the regulatory agencies to verify that the information submitted to them is accurate and complete. The FDA and other global regulatory agencies have indicated recently that they may make PAI optional, if the previous compliance or system based inspections of the applying company have been satisfactory. Upon successful inspection or verification of the information submitted, a BLA may be approved by the regulatory agencies permitting shipping of the approved product produced from the manufacturing facility to its customers.

SUMMARY

In summary, process validation for a new cell culture derived-product, process, technology, or a new manufacturing facility should be carried out with a lot of careful planning and brainstorming along with a pinch of passion and dedication to details. Often process validation projects end up in tremendous delays and exorbitant costs due to poor planning, unrealistic goals and schedules, and inexperience of the assigned staff. It is a very expensive activity and should be carried out with good planning and caution. Validation activities take time on their own and often cannot be sped up no matter how many resources are poured into it. Albeit challenging and tough, it is an unforgettable journey that more often than not results in a joyful regulatory approval of a new product, process, technology, or a new manufacturing facility. Once taken successfully, one craves for this journey again and again and again.

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(54) **INCREASE IN STRESS TOLERANCE WITH ASCORBIC ACID DURING FERMENTATION**

(76) Inventors: **Danilo Porro**, Erba (Como) (IT); **Paola Branduardi**, Milano (IT); **Diethard Mattanovich**, Wien (AT); **Michael Sauer**, Wien (AT)

Correspondence Address:
RATNERPRESTIA
P.O. BOX 1596
WILMINGTON, DE 19899 (US)

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(57) **ABSTRACT**

A method of increasing stress tolerance in recombinant organisms that have been engineered for industrial production is described. Stress tolerance is increased by making L-ascorbic acid available to the recombinant organism, either by exogenous addition to the culture medium or by endogenous production from D-glucose by the recombinant organism. To enable endogenous production, the recombinant organism is transformed with a coding region encoding a mannose epimerase (ME), a coding region encoding an L-galactose dehydrogenase (LGDH), and a D-arabinono-1, 4-lactone oxidase (ALO). The recombinant organism may be further transformed with a myoinositol phosphatase (MIP).

Figure 1

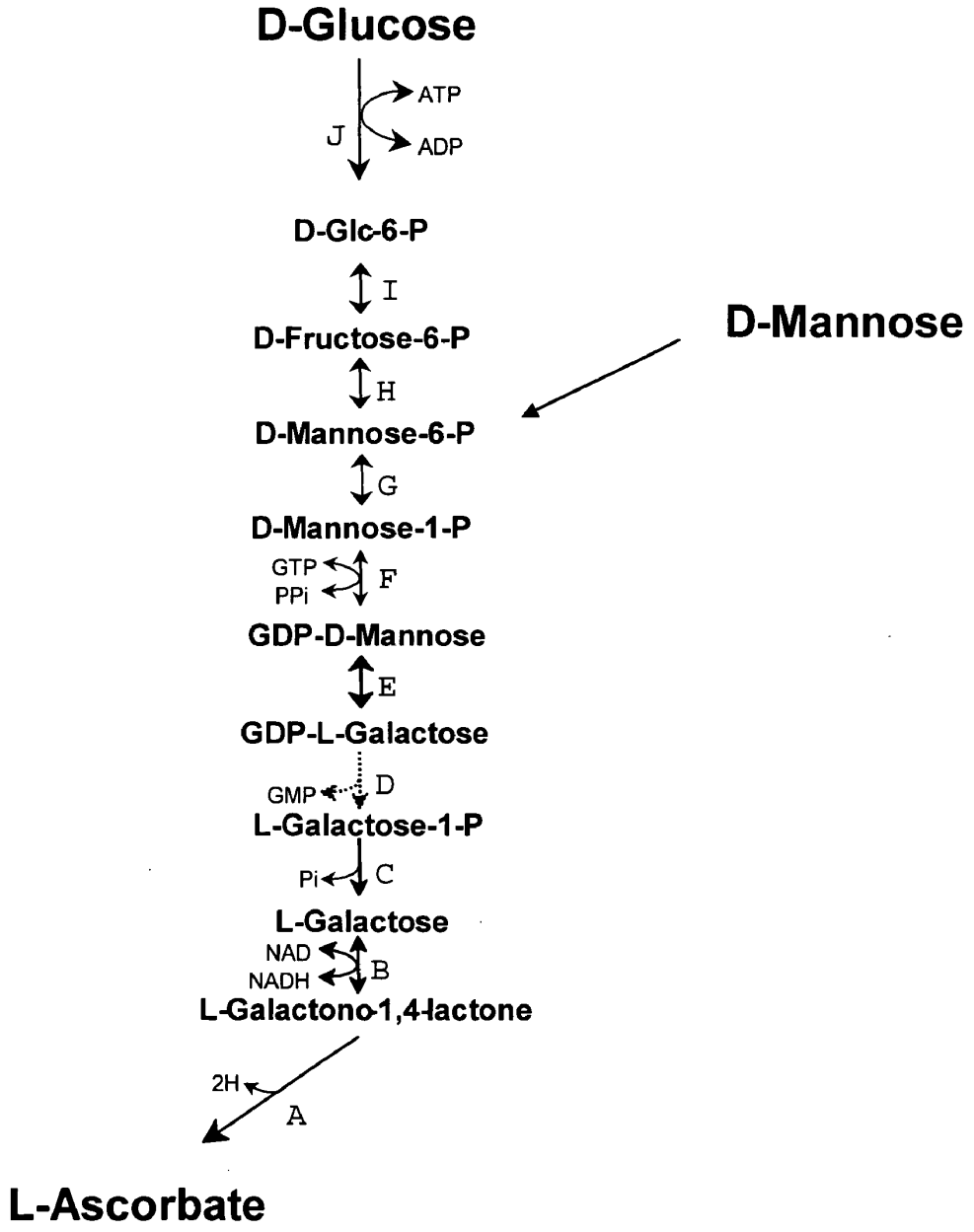

D-Glucose
ATP
J
ADP
D-Glc-6-P
I
D-Fructose-6-P
D-Mannose
H
D-Mannose-6-P
G
D-Mannose-1-P
GTP
PPi
F
GDP-D-Mannose
E
GDP-L-Galactose
GMP
D
L-Galactose-1-P
Pi
C
L-Galactose
NAD
NADH
B
L-Galactono-1,4-lactone
2H
A
L-Ascorbate

Figure 2A

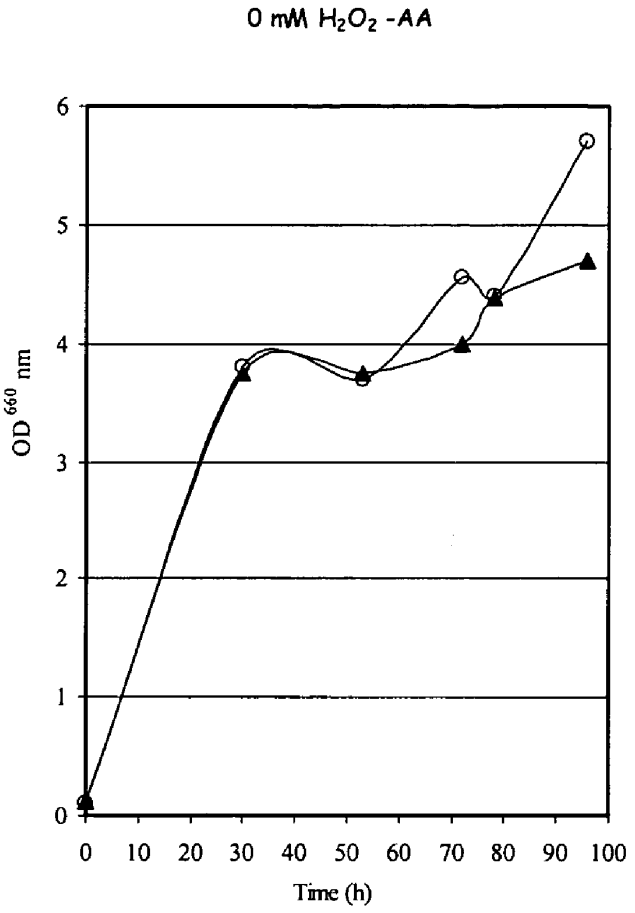

0 mM H_2O_2 -AA
OD^{660} nm
0
1
2
3
4
5
6
0 10 20 30 40 50 60 70 80 90 100
Time (h)

Figure 2B

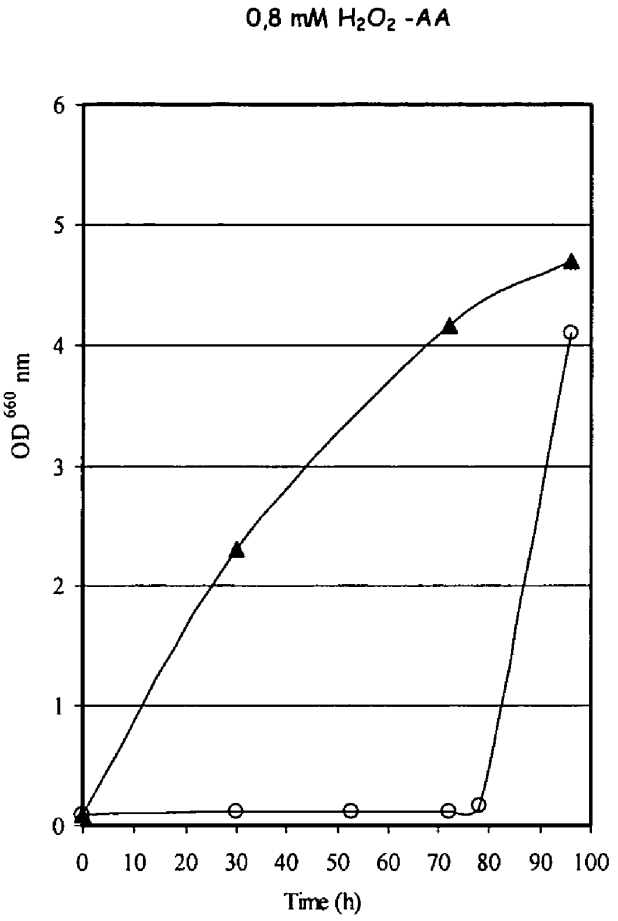

0,8 mM H_2O_2 -AA
OD^{660} nm
0
1
2
3
4
5
6
0 10 20 30 40 50 60 70 80 90 100
Time (h)

Figure 2C

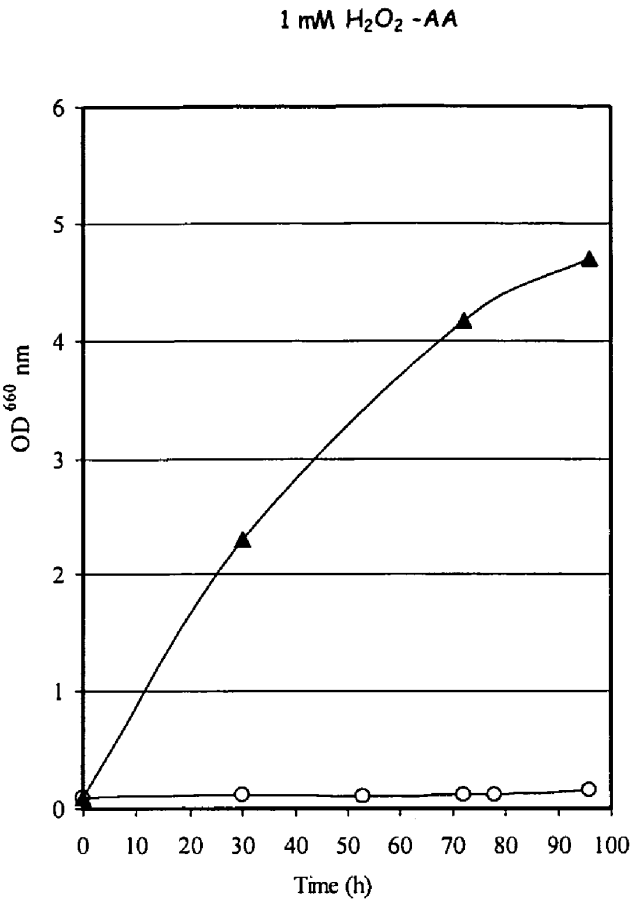

1 mM H_2O_2 -AA
6
5
4
3
2
1
0
OD^{660} nm
0
10
20
30
40
50
60
70
80
90
100
Time (h)

Figure 3A

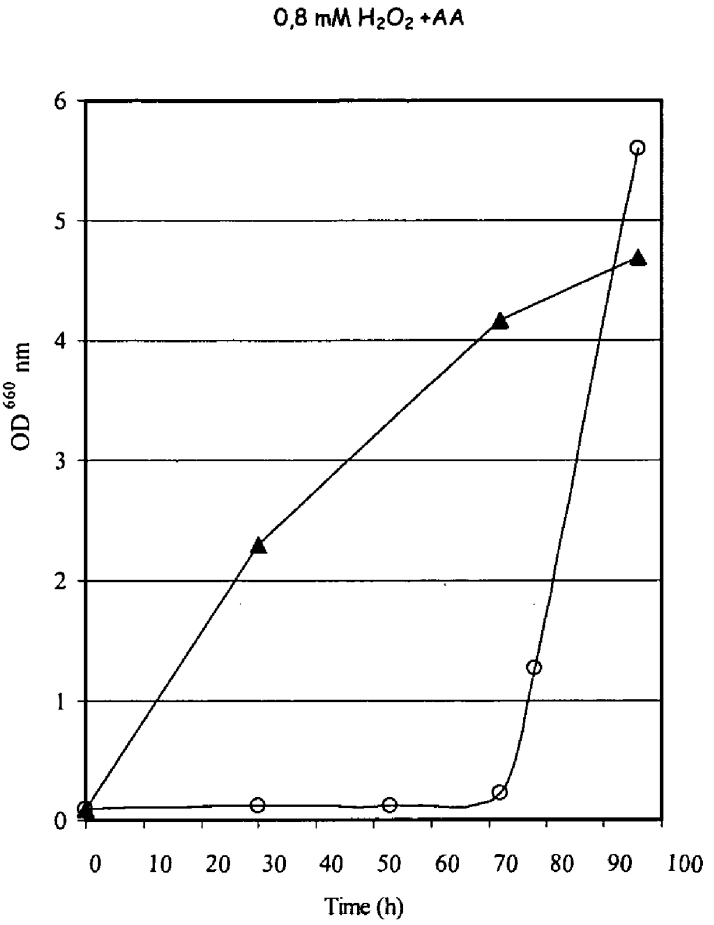

0,8 mM H_2O_2 +AA
OD^{660} nm
0
1
2
3
4
5
6
0
10
20
30
40
50
60
70
80
90
100
Time (h)

Figure 3B

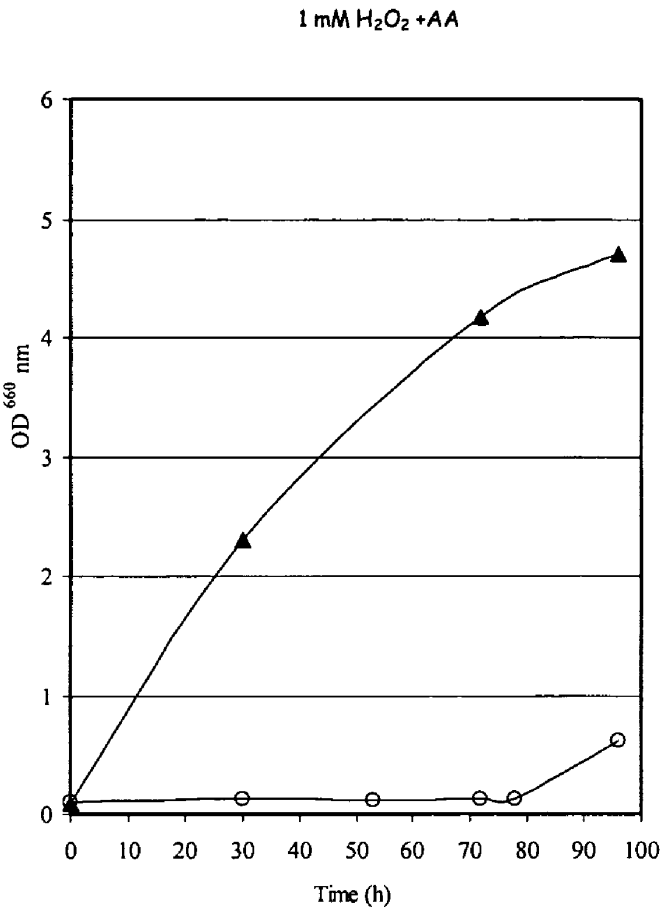
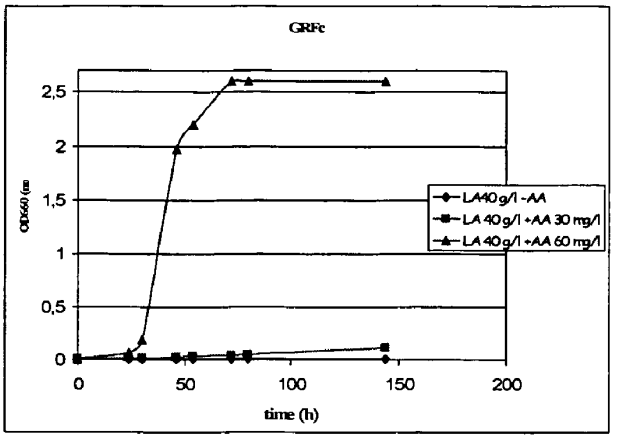
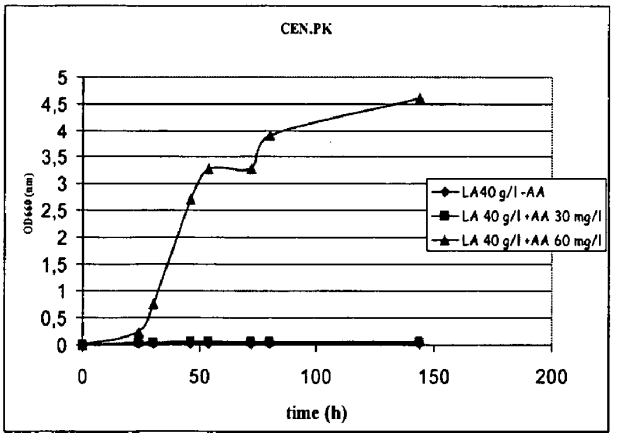

1 mM H_2O_2 +AA
OD^{660} nm
0
1
2
3
4
5
6
0
10
20
30
40
50
60
70
80
90
100
Time (h)

Figure 3C


GRFe
2,5
2
1,5
1
0,5
0
0
50
100
150
200
time (h)
LA40 g/l -AA
LA 40 g/l +AA 30 mg/l
LA 40 g/l +AA 60 mg/l


CEN.PK
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
0
50
100
150
200
time (h)
LA40 g/l -AA
LA 40 g/l +AA 30 mg/l
LA 40 g/l +AA 60 mg/l

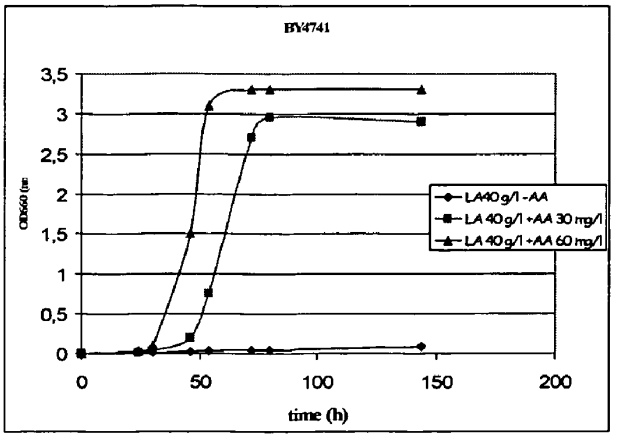

BY4741
3,5
3
2,5
2
1,5
1
0,5
0
0
50
100
150
200
time (h)
LA40 g/l -AA
LA 40 g/l +AA 30 mg/l
LA 40 g/l +AA 60 mg/l

Figure 4A

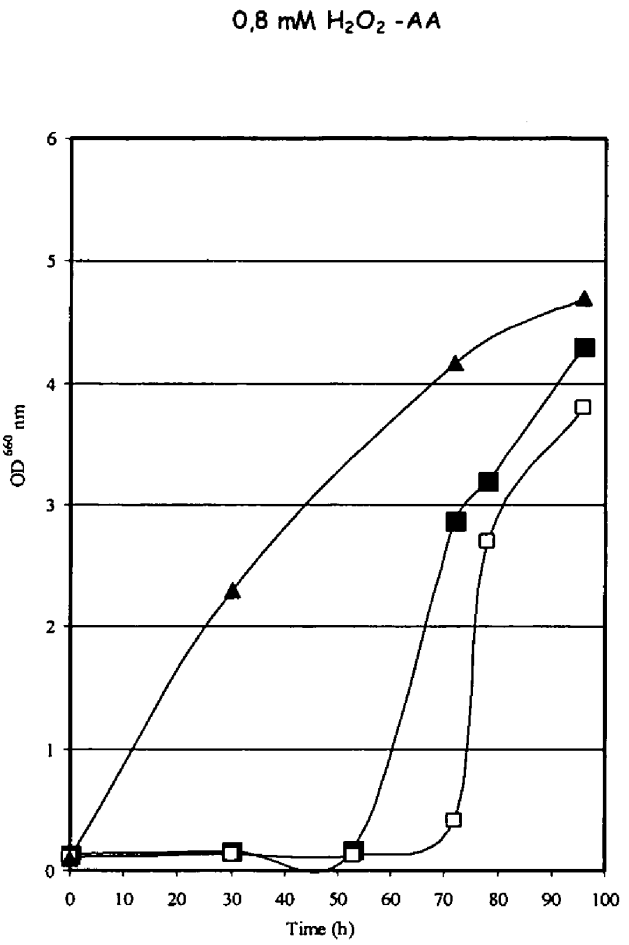

0,8 mM H_2O_2 -AA
OD^{660} nm
0
1
2
3
4
5
6
0
10
20
30
40
50
60
70
80
90
100
Time (h)

Figure 4B

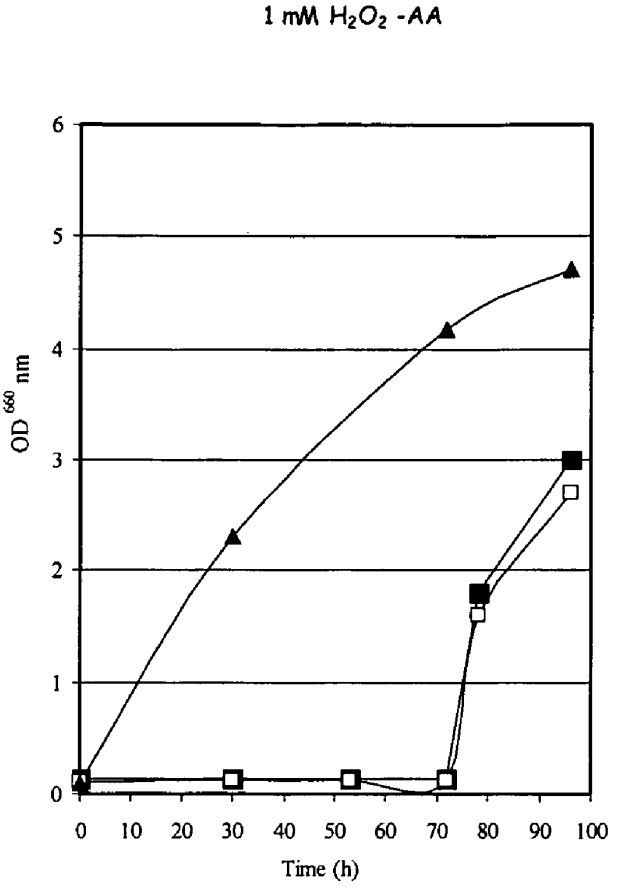

1 mM H_2O_2 -AA
OD^{660} nm
0
1
2
3
4
5
6
0 10 20 30 40 50 60 70 80 90 100
Time (h)

Figure 5A

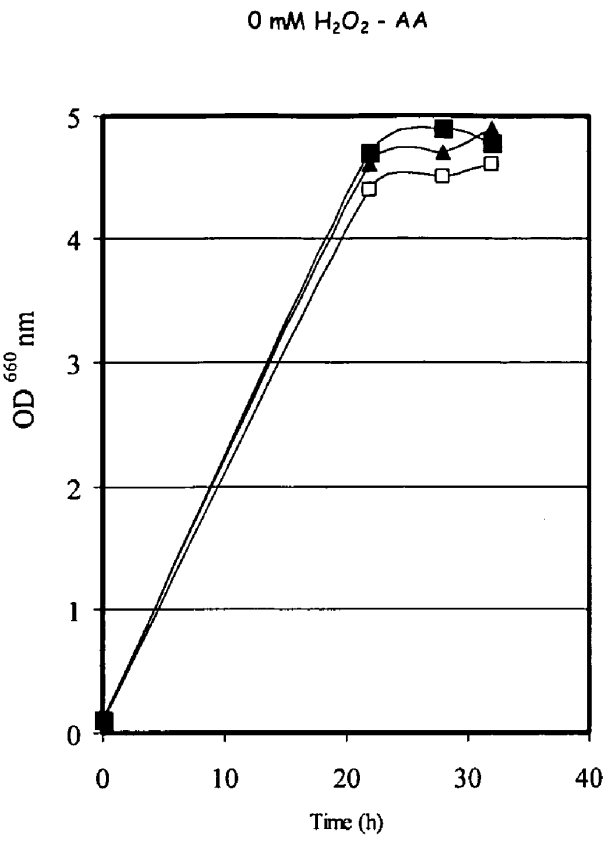


Figure 5b

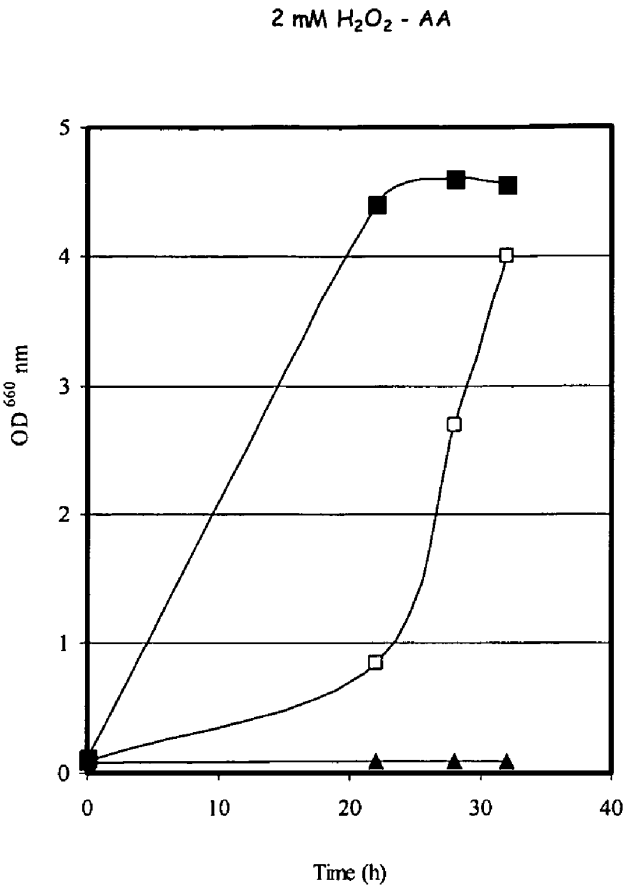


Figure 6

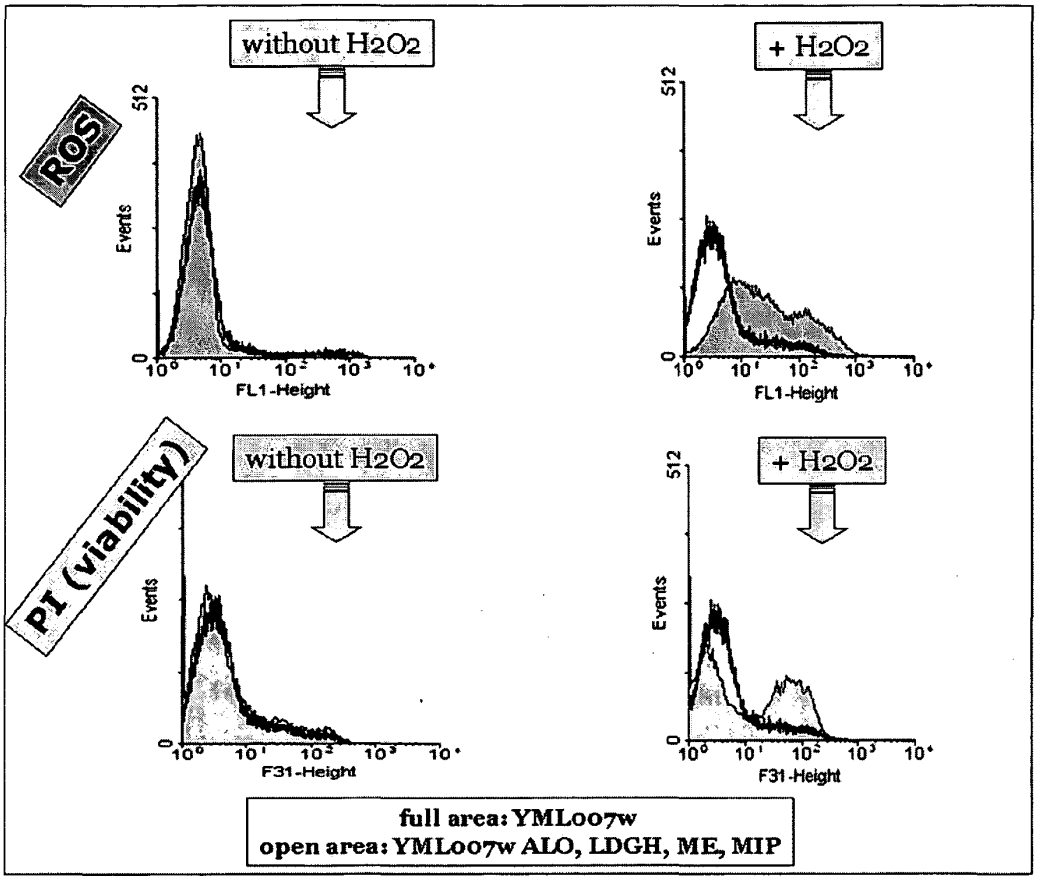
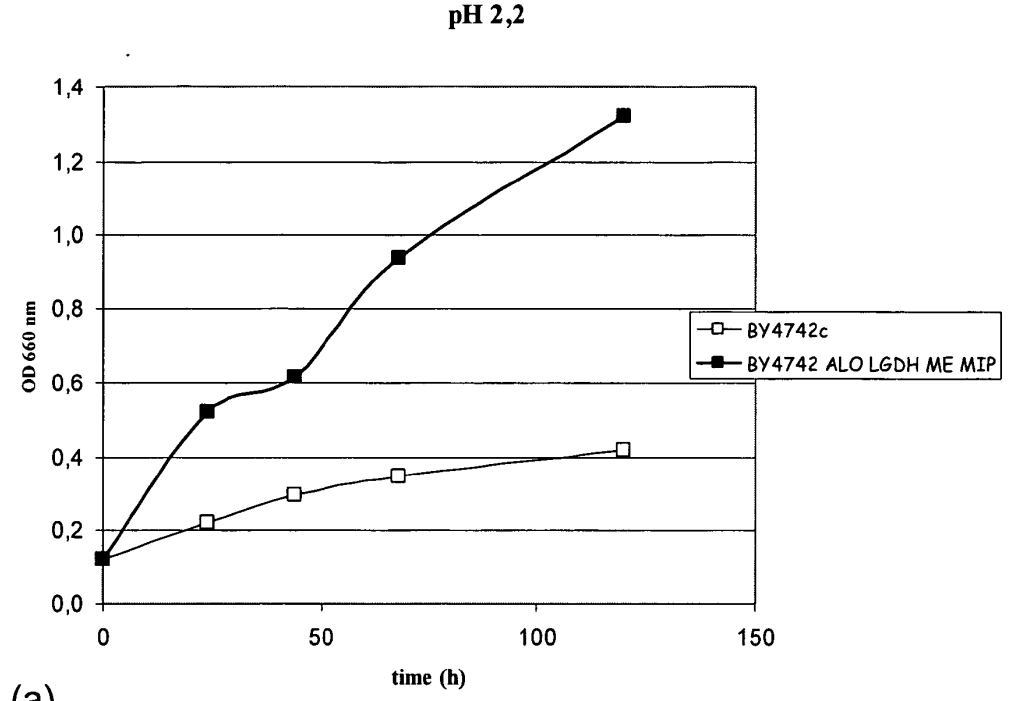
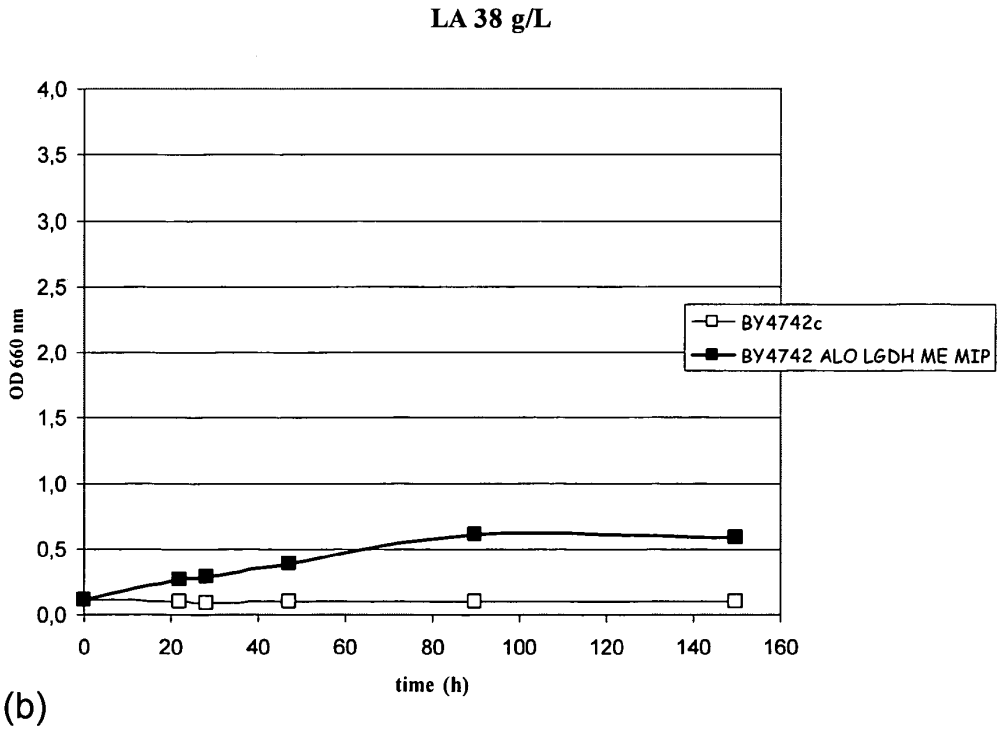

without H2O2
+ H2O2
ROS
Events
512
0
FL1-Height
without H2O2
+ H2O2
PI (viability)
F31-Height
full area: YML007w
open area: YML007w ALO, LDGH, ME, MIP

Figure 7

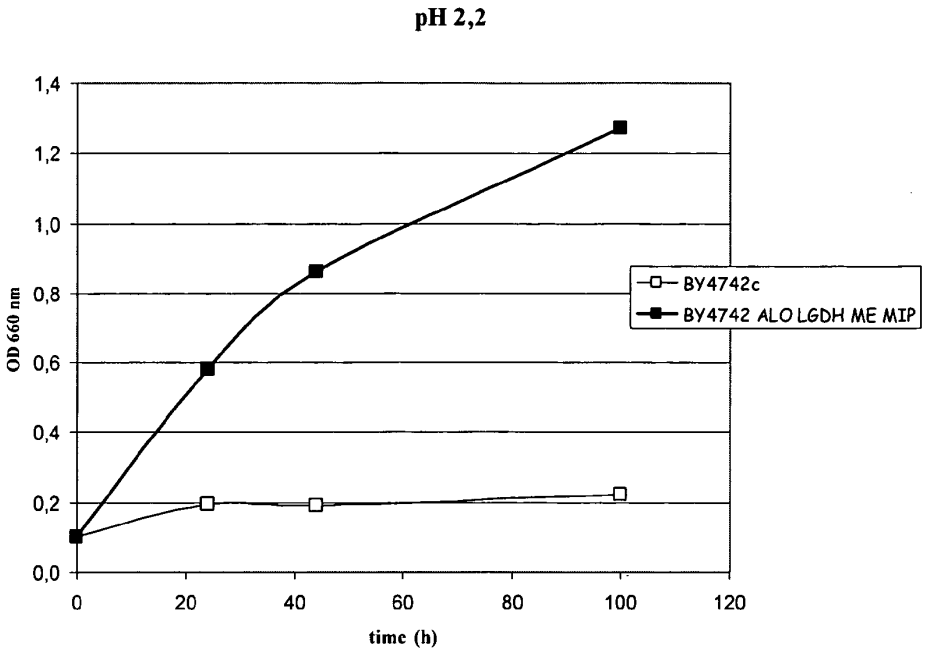

pH 2,2
OD 660 nm
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0
50
100
150
time (h)
BY4742c
BY4742 ALO LGDH ME MIP

(a)

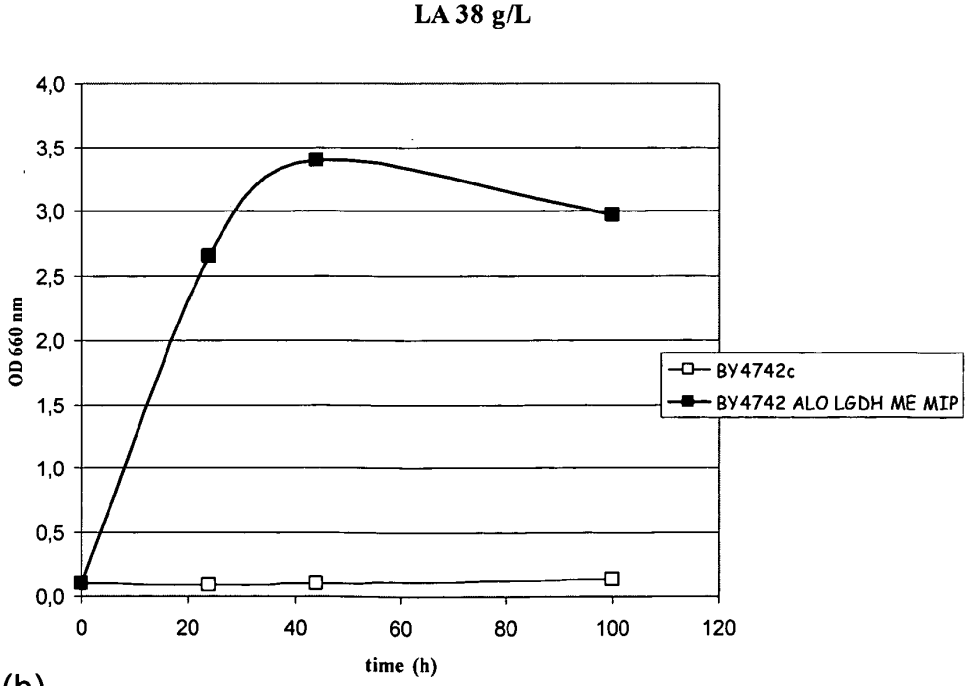

LA 38 g/L
OD 660 nm
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0
20
40
60
80
100
120
140
160
time (h)
BY4742c
BY4742 ALO LGDH ME MIP

(b)

Figure 8

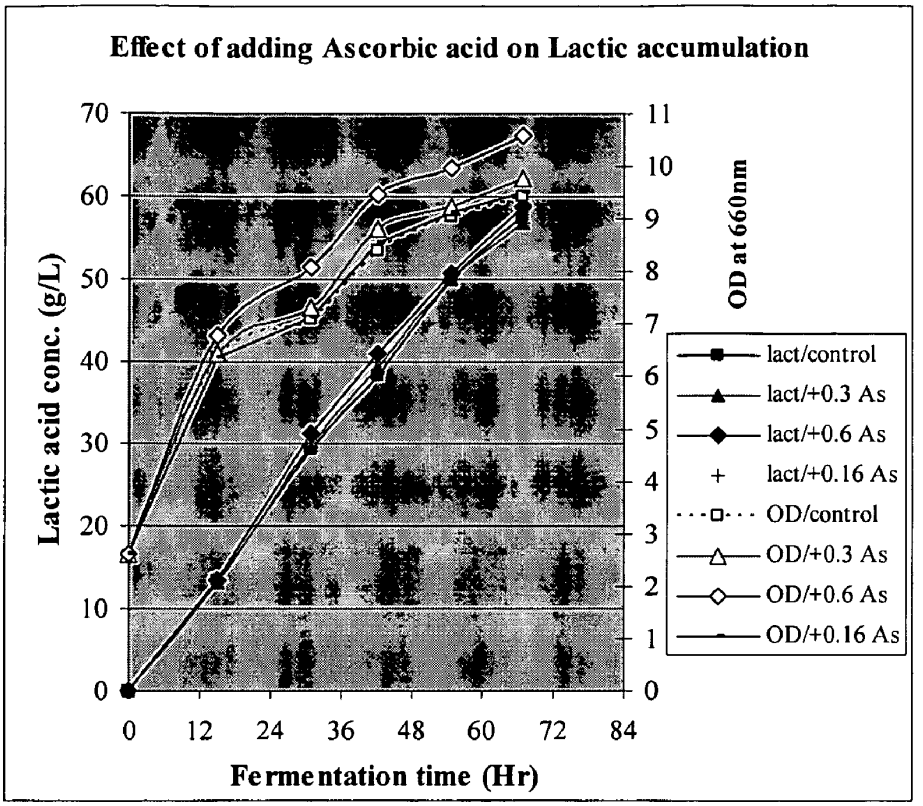

pH 2,2
OD 660 nm
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0
20
40
60
80
100
120
time (h)
BY4742c
BY4742 ALO LGDH ME MIP

(a)


LA 38 g/L
OD 660 nm
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0
20
40
60
80
100
120
time (h)
BY4742c
BY4742 ALO LGDH ME MIP

(b)

Figure 9


Effect of adding Ascorbic acid on Lactic accumulation
Lactic acid conc. (g/L)
OD at 660nm
Fermentation time (Hr)
0
10
20
30
40
50
60
70
0
1
2
3
4
5
6
7
8
9
10
11
0
12
24
36
48
60
72
84
lact/control
lact/+0.3 As
lact/+0.6 As
lact/+0.16 As
OD/control
OD/+0.3 As
OD/+0.6 As
OD/+0.16 As

INCREASE IN STRESS TOLERANCE WITH ASCORBIC ACID DURING FERMENTATION

RELATED APPLICATIONS

[0001] This application is a continuation-in-part of U.S. Ser. No. 11/105,162, filed on Apr. 13, 2005, which is incorporated herein by reference. This application claims priority from U.S. Ser. No. 11/105,162 and from PCT/US06/012854, filed on Apr. 7, 2006, also incorporated herein by reference.

FIELD OF THE INVENTION

[0002] The present invention relates generally to the field of increasing stress tolerance in organisms used for industrial production. More particularly, it relates to a process for making L-ascorbic acid available to organisms during industrial production.

BACKGROUND

[0003] Microorganisms and cells can be easily grown on an industrial scale and are frequently employed in the commercial production of compounds such as organic acids, amino acids, vitamins, polyols, solvents, biofuels, therapeutics, vaccines, proteins, and peptides. Both prokaryotic and eukaryotic microorganisms are today easily and successfully used for the production of heterologous proteins as well as for the production of natural or engineered metabolites. Among prokaryotes, *Escherichia coli* and *Bacillus subtilis* are often used. Among eukaryotes, the yeasts, *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, are often used. However, in an industrial process, wherein the organism is used as a means for production, stress on the organism typically leads to lower or zero production of the product, lower or zero productivity, a lower or zero yield of the product, or two or more thereof. Bacteria, yeast, other fungi, cultured animal cells, and cultured plant cells show similar responses to stress. (Close, D. C., et al., *Oxidative Stress, Exercise, and Aging*, H. M. Alessio, A. E. Hagerman, Eds. (2006), pp. 9-23; Sugiyama, K., et al., (2000), J Biol. Chem. 275, 15535-15540; Mongkolsuk, S. and Helmann, J. D. (2002), Molecular Microbiology 45, 9-15). Techniques for minimizing stress would therefore be useful for improving industrial production by these organisms.

[0004] Stresses may have cellular (internal or intracellular) origins, environmental (external or extracellular) origins, or both. Classical examples of the internally-originating stresses include protein and metabolite overproduction (in terms of weight/volume) and protein and metabolite overproductivity (in terms of weight/volume per unit time), among others. Examples of externally-originating stresses include high osmolarity, high salinity, oxidative stress, high or low temperature, non-optimal pH, presence of organic acids, presence of toxic compounds, and macro- and micronutrient starvation.

[0005] Stress is typically caused by stressors (or stimuli). Stressors are negative influences on a cell that require the cell to dedicate more effort to maintain equilibrium than is required in the absence of the stressor. This greater effort can lead to a higher or lower metabolic activity, lower growth rate, lower viability, or lower productivity, among other effects. Stressors are agents of a physical, chemical or biological nature that represent a change in the usual intracellular or extracellular conditions for any given life form. It follows that while a specific condition (e.g., a temperature of 65° C.) may be stressful (or even lethal) to a certain species that normally lives at 37° C., it may be optimal for a thermophilic organism.

[0006] At the cellular level, stress can damage DNA, lipids, proteins, membranes, and other molecules and macromolecules, induce apoptosis (programmed cell death), cell necrosis and cell lysis, and impair cell integrity and cell viability. These effects are often mediated by the generation of reactive oxygen species (ROS).

[0007] ROS can be generated through both intracellular and extracellular stimuli. The majority of endogenous ROS are produced through leakage of these species from the mitochondrial electron transport chain. In addition, cytosolic enzyme systems, including NADPH oxidases and by-products of peroxisomal metabolism, are also endogenous sources of ROS. Generation of ROS also can occur through exposure to numerous exogenous agents and events including ionizing radiation, UV light, chemotherapeutic drugs, environmental toxins, and hyperthermia. Oxidative damage caused by intracellular ROS can result in DNA base modifications, single- and double-strand DNA breaks, and the formation of apurinic/apyrimidinic lesions, many of which are toxic and/or mutagenic. Therefore, the resulting DNA damage may also be a direct contributor to deleterious biological consequences (Tiffany, B. et al., (2004) Nucleic Acids Research 32, 3712-3723).

[0008] One example of an industrial process known to be hampered by stress responses is the production of lactic acid by bacteria or yeast. During a typical lactic acid fermentation, the accumulation of lactic acid in the medium also causes a drop in pH of the medium. The stress of low pH is amplified by the ability of the organic free acid to diffuse through the membrane and dissociate in the higher pH of the cytoplasm. The accumulation of lactic acid inhibits cell growth and metabolic activity. The toxicity of these stresses is mediated at least in part by reactive oxygen species. As a result, the extent of lactic acid production is greatly reduced by the accumulation of lactic acid in the medium.

[0009] The addition of $Ca(OH)_2$, $CaCO_3$, NaOH, or NH_4OH to the fermentation medium to neutralize the lactic acid and to thereby prevent the pH drop is a conventional operation in industrial processes to counteract the negative effects of free lactic acid accumulation. These processes allow the production of lactate(s) by maintaining the pH at a constant value in the range of about 5 to 7, which is well above the pKa of lactic acid (3.86).

[0010] However, this neutralization procedure has major disadvantages. Additional operations are required to regenerate free lactic acid from its salt and to dispose of or recycle the neutralizing cation, which adds expense to the process. The added operations and expense could be lessened if free lactic acid could be accumulated by organisms growing at low pH values. To this end, the use of recombinant yeast that are engineered for industrial production of free lactic acid, and, in particular, recombinant yeast from strains showing greater tolerance for extreme environmental conditions have been described. Engineered strains of recombinant yeast functionally transformed with a gene for lactate dehydrogenase (LDH) in the genera *Saccharomyes, Zygosaccharomyces, Torulaspora*, and *Kluveromyces* have been produced as

described in U.S. Pat. Nos. 6,429,006 and 7,049,108. While these recombinant strains show improved efficiency of lactic acid production at low pH, they are still adversely affected by stresses. In addition, it may be necessary to use organisms or strains that are less tolerant of extreme environmental conditions for the industrial production of specific compounds.

[0011] Ascorbic acid is a known antioxidant that is produced in all higher plants and many higher animals. Ascorbic acid has been shown to modulate the heat shock response in yeast through an effect on ROS(C. Moraitis and B. P. G. Curran. (2004), *Yeast* 21, 313-323), and to improve cell viability and reduce proteolysis of the end product of high cell-density fermentation (Xiao, A. et al. (2006), Appl. Microbiol. Biotechnol. 72, 837-844). These effects suggest that ascorbic acid could improve stress tolerance in general in organisms utilized for industrial production.

[0012] We have shown that recombinant yeast that are functionally transformed to produce L-ascorbic acid, the biologically active enantiomer, from D-glucose produce lower levels of ROS and exhibit improved growth and viability under conditions of low pH, oxidative stress, and in the presence of high concentrations of lactic acid. (Branduardi, P., et al., International Specialised Symposium on Yeast. ISSY25, Systems Biology of Yeast—From Models to Applications. "L-ascorbic acid production from D-glucose in metaboloic engineered *Saccharomyces cerevisiae* and its effect on strain robustness." Hanasaari, Espoo, Finland, Jun. 21, 2006).

[0013] Accordingly, it would be advantageous to industrial fermentation processes if the organisms and cells used for industrial production could endogenously produce L-ascorbic acid from D-glucose.

SUMMARY OF THE INVENTION

[0014] The present invention relates to a method of increasing stress tolerance in a recombinant organism that is engineered for industrial production of at least one product. The method comprises making L-ascorbic acid available to the recombinant organism.

[0015] In one embodiment, ascorbic acid is made available by functionally transforming the recombinant organism with a coding region encoding a mannose epimerase (ME), a coding region encoding an L-galactose dehydrogenase (LGDH), and a coding region encoding a D-arabinono-1,4-lactone oxidase (ALO). In a further embodiment, the functionally transformed, recombinant organism is further functionally transformed with a coding region encoding a myoinositol phosphatase (MIP).

[0016] In another embodiment, the L-ascorbic acid is made available by culturing the recombinant organism in culture medium containing an effective amount of L-ascorbic acid.

DESCRIPTION OF THE DRAWINGS

[0017] FIG. **1** shows the main plant pathway for the synthesis of L-ascorbic acid from D-glucose.

[0018] FIG. **2** shows the optical density at 660 nm of BY4742 (▲) and YML007w (yap1 mutant strain) (○) yeast in the absence (FIG. **2***a*) and presence (FIGS. **2***b*-**2***c*) of oxidative stress. Yap1 activates genes required for the response to oxidative stress; deletion of this gene leads to the observed phenotype.

[0019] FIG. **3** shows the impact of two stressors on yeast growth. FIGS. **3***a*-**3***b* show the optical density at 660 nm of BY4742 wt (▲) and YML007w (○) yeast in the presence of H_2O_2 in medium +/−ascorbic acid. FIG. **3***c* shows the optical density at 660 nm of wild type yeast GRFc, CEN.PK 113-5D, and BY4741 in the presence of 40 g/l lactic acid and zero, or increasing levels of ascorbic acid.

[0020] FIG. **4** shows the optical density at 660 nm of BY4742 wt (▲); YML007w expressing ALO, LDGH and ME (□); and YML007w expressing ALO, LDGH, ME and MIP (■) yeasts in the presence of oxidative stress (FIGS. **4***a*-**4***b*).

[0021] FIG. **5** shows the optical density at 660 nm of wild type GRFc (▲); GRF18U expressing ALO, LDGH and ME (□); and GRF18U expressing ALO, LDGH, ME and MIP (■) yeast strains in the absence (FIG. **5***a*) and presence (2 mM of H_2O_2) of oxidative stress. (FIG. **5***b*).

[0022] FIG. **6** shows ROS (upper panels) and viability (bottom panels) determination by flow cytometric analyses of *S. cerevisiae* cells producing (YML007w ALO, LDGH, ME, MIP, open area) or not producing (YML007w, full area) ascorbic acid when grown in minimal glucose medium in the presence (right) or absence (left) of hydrogen peroxide.

[0023] FIG. **7** shows growth curves of strains BY4742c (□) and BY4742 ALO, LDGH, ME, MIP (■) inoculated in minimal glucose medium at pH 2.2 (a), or in minimal glucose medium pH 3.0 containing 38 g/l of lactic acid (b).

[0024] FIG. **8** shows growth curves of strains BY4742c (□) and BY4742 ALO, LDGH, ME, MIP (■) that were first grown for 24 h in minimal glucose medium under nonlimiting conditions, and then transferred to minimal glucose medium at pH 2.2 (a), or to minimal glucose medium pH 3 containing 38 g/l of lactic acid (b).

[0025] FIG. **9** shows growth curves, as measured by OD660, and lactic acid production by *S. cerevisiae* strain NRRL Y-30696 grown in minimal glucose medium containing 2.78 g/L $CaCO_3$ and increasing concentrations of ascorbic acid (AA). 0 g/L AA (□), 0.16 g/L AA (+), 0.3 g/L AA (▲), or 0.6 g/L (♦)

DETAILED DESCRIPTION

[0026] The present invention relates to a method of increasing stress tolerance in recombinant cells or organisms that have been engineered for the industrial production of products such as organic acids, amino acids, vitamins, polyols, solvents, biofuels, therapeutics, vaccines, proteins, and peptides by increasing the available amount of ascorbic acid.

[0027] A "recombinant" cell or organism is one that contains a nucleic acid sequence that is not naturally occurring in that cell or organism, or one that contains an additional copy or copies of an endogenous nucleic acid sequence, wherein the nucleic acid sequence is introduced into the cell or organism or into an ancestor cell thereof by human action. Introduction of the gene into the cell or organism is known as "transformation" and the recipient organism or cell is said to be "transformed." Recombinant DNA techniques are

well-known to those of ordinary skill in the art, who will also understand how to choose appropriate vectors and promoters for the transformation of particular organisms or strains. (For example, see methods in Sambrook, J. and Russell, D. W., *Molecular Cloning: A Laboratory Manual,* 3rd Edition, Cold Spring Harbor Laboratory Press, 2001). Very basically, a coding region of the homologous and/or heterologous gene is isolated from a "donor" organism that possesses the gene. The recombinant organism, as well as the donor, may be a prokaryote, such as a bacterium, or a eukaryote, such as a protozoan, alga, fungus, plant, or animal.

[0028] In one well-known technique, a coding region is isolated by first preparing a genomic DNA library or a cDNA library, and second, identifying the coding region in the genomic DNA library or cDNA library, such as by probing the library with a labeled nucleotide probe that is at least partially homologous with the coding region, determining whether expression of the coding region imparts a detectable phenotype to a library microorganism comprising the coding region, or amplifying the desired sequence by PCR. Other techniques for isolating the coding region may also be used.

[0029] Methods for preparing recombinant nucleotides and transferring them into a host organism are well-known to those of ordinary skill in the art. Briefly, the desired coding region is incorporated into the recipient organism in such a manner that the encoded protein is produced by the organism in functional form. That is, the coding region is inserted into an appropriate vector and operably linked to an appropriate promoter on the vector. If necessary, codons in the coding region may be altered, for example, to create compatibility with codon usage in the target organism, to change coding sequences that can impair transcription or translation of the coding region or stability of the transcripts, or to add or remove sequences encoding signal peptides that direct the generated protein to a specific location in or outside the cell, e.g., for secretion of the protein. Any type of vector, e.g., integrative, chromosomal, or episomal, may be used. The vector may be a plasmid, cosmid, yeast artificial chromosome, virus, or any other vector appropriate for the target organism. The vector may comprise other genetic elements, such as an origin of replication to allow the vector to be passed on to progeny cells of the host carrying the vector, sequences that facilitate integration into the host genome, restriction endonuclease sites, etc. Any promoter active in the selected organism, e.g., homologous, heterologous, constitutive, inducible, or repressible may be used. An "appropriate" vector or promoter is one that is compatible with the selected organism and will generate a functional protein in that organism. The recombinant organism thus transformed is referred to herein as being "functionally transformed."

[0030] The recombinant cells and organisms of the invention can be obtained by any method allowing a foreign DNA to be introduced into a cell, for example, transformation, electroporation, conjugation, fusion of protoplasts or any other known technique (Spencer J. F. et al. (1988), Journal of Basic Microbiology 28, 321-333). A number of protocols are known for transforming yeast, bacteria, and eukaryotic cells. Transformation can be carried out by treating the whole cells in the presence of lithium acetate and of polyethylene glycol according to Ito H. et al. ((1983), J. Bacteriol., 153:163), or in the presence of ethylene glycol and dimethyl sulphoxyde according to Durrens P. et al. ((1990) Curr. Genet., 18:7). An alternative protocol has also been described in EP 361991. Electroporation can be carried out according to Becker D. M. and Guarente L. ((1991) Methods in Enzymology, 194:18). The use of non-bacterial integrative vectors may be preferred when the yeast biomass is used at the end of the fermentation process as stock fodder or for other breeding, agricultural or alimentary purposes.

[0031] The transformed organism is propagated in an appropriate culture medium. Culturing techniques and specialized media are well known in the art. For industrial production, the organism is preferably cultured in an appropriate medium in a fermentation vessel.

[0032] Organisms frequently utilized for industrial production are yeast and bacteria. Yeast to be transformed can be selected from any known genus and species of yeast. Yeast species are described by N. J. W. Kreger-van Rij, ("The Yeasts," (1987) *Biology of Yeasts*, A. H. Rose and J. S. Harrison, Eds. London: Academic Press, Chapter 2) In one embodiment, the yeast genus is selected from the group consisting of *Saccharomyces, Zygosaccharomyces, Candida, Hansenula, Kluyveromyces, Debaromyces, Nadsonia, Lipomyces, Torulopsis, Kloeckera, Pichia, Schizosaccharomyces, Trigonopsis, Brettanomyces, Cryptococcus, Trichosporon, Aureobasidium, Lipomyces, Phaffia, Rhodotorula, Yarrowia*, and *Schwanniomyces*. In another embodiment, the yeast is selected from *S. cerevisiae* strains, including GRF18U, W3031B, BY4742 (MATα; his3; leu2, lys2; ura3, EuroScarf Accession No. Y10000); *Z. bailii* ATCC 60483; *K. lactis* PM6-7A; BY4741 (MATα; his3; leu2; met15; ura3, Euroscarf Accession No. Y00000), CEN.PK 113-5D (MATα ura3-52; cir+), and yeast strains engineered to produce lactic acid, including NRRL Y-30696, NRRL Y-30698, NRRL Y-30742; *K. lactis* PM6-7/pEPL2, PMI/C1[pELP2]; *Zygosaccharomyces bailii* ATTC36947/pLAT-ADH, ATCC60483/pLAT-ADH.

[0033] Yeast have been widely utilized in the production of products. Yeast biomass is an important product as cultures for development of food products as well as a nutrient rich food and feed component. Genetic engineering has broadened the value of yeast production systems providing a route to organic acids (Porro, D. et al. (2002), U.S. Pat. No. 6,429,006); vitamins (Shiuan, D., US2003/0104584); polyols (Geertman, J. M, et al., (2006) Metabolic Engineering, June 30:(Epublication); biofuel (Ho, N. W. Y. and Tsao, G. T. (1998), U.S. Pat. No. 5,789,210); (Bosman, F., et al. (2006) U.S. Pat. No. 7,048,930); proteins (Gerard, G. F., et al. (2006). U.S. Pat. No. 7,115,406); and peptides (Lee, S. Y., et al., Lett. Appl. Microbiol (2003), 36, 121-128.).

[0034] Bacteria to be transformed can be selected from any known genus and species of the Eubacteria or the Archaea (also encompassed herein by the term, "bacteria"). Bacteria are cataloged at the NCBI Taxonomy website: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Taxonomy. In one embodiment the bacteria can be selected from the genera *Bacillus, Escherichia, Lactobacillus, Lactococcus, Pseudomonas*, or *Acetobacter.*

[0035] Bacteria have been widely utilized to produce industrial products. The natural range of available products has been extended by mutagenesis and screening and further by genetic engineering. Bacteria provide routes to organic acids (WO2006/083410); amino acids (WO2005/090589);

vitamins (Santos, et al., Abstracts of Papers, 232nd ACS National Meeting, San Francisco, Calif., United States, Sep. 10-14, 2006, BIOT-243); polyols (Dunn-Coleman, N. S., et al. (2006) U.S. Pat. No. 7,074,608); solvents (Harris, L. M., et al. (2001), Journal of Industrial Microbiology & Biotechnology 27, 322-328); biofuels (Ingram, L. O. and Zhou, S. WO2000/071729); therapeutics (Pizza, M., et al. (2006) U.S. Pat. No. 7,115,730); proteins (Gerard, G. F., et al. (2006) U.S. Pat. No. 7,115,406); and peptides (Knapp, S., et al. (1992) U.S. Pat. No. 5,159,062).

[0036] Filamentous fungi are widely utilized to produce organic acids (Bizukojc, M. and Ledakowicz, S., Process Biochemistry (2004), 39, 2261-2268.); and proteins (Wang, L., et al., (2003) Biotechnology Advances 23, 115-129). Filamentous fungi to be transformed can be selected from any known genus and species. Fungi are cataloged at the NCBI Taxonomy Website: http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=4751.

[0037] In one embodiment the filamentous fungi can be selected from the genera *Rhizopus, Aspergillus*, or *Trichoderma.*

[0038] In one embodiment of the invention, the recombinant organism is functionally transformed with coding regions that encode a mannose epimerase (D-mannose:L-galactose epimerase; ME), L-galactose dehydrogenase (LGDH); and D-arabinono-1,4-lactone oxidase (ALO). These coding sequences enable the recombinant organism to produce enzymes necessary for the endogenous production of L-ascorbic acid from D-glucose. As a result of transformation with ME, LGDH, and ALO, and endogenous production of L-ascorbic acid, the organism shows increased tolerance to stress when compared with a strain of the same organism that cannot produce L-ascorbic acid.

[0039] An ME is any GDP-mannose-3,5-epimerase (5.1.3.18), that is any enzyme that catalyzes the conversion of GDP-mannose to GDP-L-galactose (FIG. **1**). An exemplary ME is encoded by the sequence listed as SEQ ID NO:1.

[0040] In one embodiment, the ME has at least about 95% identity with SEQ ID NO:1. "Identity" can be determined by a sequence alignment performed using the ClustalW program and its default values, namely: DNA Gap Open Penalty=15.0, DNA Gap Extension Penalty=6.66, DNA Matrix=Identity, Protein Gap Open Penalty=10.0, Protein Gap Extension Penalty=0.2, Protein matrix=Gonnet. Identity can be calculated according to the procedure described by the ClustalW documentation: "A pairwise score is calculated for every pair of sequences that are to be aligned. These scores are presented in a table in the results. Pairwise scores are calculated as the number of identities in the best alignment divided by the number of residues compared (gap positions are excluded). Both of these scores are initially calculated as percent identity scores and are converted to distances by dividing by 100 and subtracting from 1.0 to give number of differences per site. We do not correct for multiple substitutions in these initial distances. As the pairwise score is calculated independently of the matrix and gaps chosen, it will always be the same value for a particular pair of sequences."

[0041] In another embodiment, the recombinant organism transformed with the coding sequences for ME, LGDH, and ALO is further functionally transformed with a coding region encoding a myoinositol phosphatase (MIP). An MIP is any myoinositol phosphatase (3.1.3.25), that also catalyzes the conversion of L-galactose-1P to L-galactose. L-galactose-1-phosphatase has been annotated as inositol/myoinositol monophosphatase galactose-1-phosphatase and may be referred to as MIP/VTC4 (Conklin, P. L. et al. (2006) J. Biol. Chem. 281, 15662-70). In one embodiment, the MIP has at least about 95% identity with SEQ ID NO:2. Identity is determined as described above.

[0042] In another embodiment, the recombinant organism is further transformed with a coding region encoding an enzyme selected from L-galactono-1,4-lactone dehydrogenase (AGD), D-arabinose dehydrogenase (ARA) or L-gulono-1,4-lactone oxidase (GLO), as described, for example, in U.S. Pat. No. 6,630,330, which is incorporated herein by reference.

[0043] Although the pathway for the production of ascorbic acid in plants is shown in FIG. **1**, the present invention is not limited to the enzymes of the pathways known for the production of L-ascorbic acid intermediates or L-ascorbic acid in plants, yeast, or other organisms. (Examples of known L-ascorbic acid pathways in plants and animals are described in Conklin, P. L., et al. (2006), J. Biol. Chem. 281, 15662-15670; and in Valpuesta, V. and Botella, M. A. (2004) Trends in Plant Science 12, 573-577). One of ordinary skill in the art will understand that increasing flux through any pathway resulting in L-ascorbic acid biosynthesis will result in production of higher levels of L-ascorbic acid. This can be accomplished by increasing the levels of enzymes in the pathway that are limiting.

[0044] The coding regions for any of the desired enzymes may be isolated from any source or may be chemically synthesized. Following transformation with the coding regions for ME, LGDH, and ALO, (with or without the coding region for MIP), the recombinant organism is cultured in medium containing a carbon source that can be converted to L-ascorbic acid, such as D-glucose.

[0045] When the recombinant organism for industrial production is a eukaryotic organism, it is important to ensure that each of the enzymes used to produce ascorbic acid is appropriately compartmentalized in the eukaryotic cell. This is accomplished by including sequences encoding targeting labels in the recombinant vector. These types of sequences are disclosed, for example, in Alberts, B., et al., *Molecular Biology of the Cell,* 4th Edition, New York: Garland Science Publ., 2002, pages 659-710.

[0046] With respect to the invention, "production" means the process of making one or more products using a recombinant organism. Production can be quantified at any moment in time after commencement of the process by determining the weight of a product produced per weight or volume of the medium on which the recombinant organism's growth and survival is maintained, or weight or volume of the recombinant organism's biomass. "Productivity" means the amount of production, as quantified above, over a given period of time (e.g., a rate such as g/L per hour, mg/L per week, or g/g of biomass per hour). "Yield" is the amount of product produced per the amount of substrate converted into the product. This definition of "yield" also applies to endogenous production of L-ascorbic acid.

[0047] Stress tolerance, as used herein, may manifest as a decrease in the negative impact of stress on the organism,

such as a decline in the production of ROS or a positive effect on productivity, yield, or production. An increase in stress tolerance can be measured by a number of parameters, for example, as an increase in growth rate, an increase in cell density, a decrease in the inhibition of productivity, an increase in viability, an increase in metabolism, or an increase in yield, productivity, or production. An "effective amount" of L-ascorbic acid is an amount of L-ascorbic acid present in the culture medium that gives rise to an improvement in stress tolerance as measured by any of these parameters, when compared with stress tolerance of the organism grown in medium that does not contain L-ascorbic acid.

[0048] As shown in FIGS. **2-5**, yeast transformed with coding sequences for ME, LGDH, and ALO, or with this group of coding sequences plus a coding sequence for MIP, have greater stress tolerance than yeast that are not so transformed. FIG. **7** shows that endogenously produced L-ascorbic acid correlates with increased tolerance to low pH and oxidative stresses. This increased stress resistance can manifest as one or more of increased growth rate of the transformed organism, increased viability of the transformed organism, or increased production by the transformed organism.

[0049] We also show, in FIG. **3**, that the addition of L-ascorbic acid to the fermentation medium improves stress tolerance, in particular, tolerance to low pH and oxidative stress. Accordingly, in one embodiment of the invention, the available amount of ascorbic acid is increased by adding L-ascorbic acid to the fermentation medium. Exogenous L-ascorbic acid may be added to cultures that do or do not produce L-ascorbic acid endogenously.

[0050] Though not wishing to be bound by a single theory, we suggest that the increased stress tolerance results from an increase in antioxidant levels (specifically, L-ascorbic acid) and a reduction in the levels of endogenous reactive oxygen species (ROS) in the organism, imparting greater resistance to oxidative stress, as shown in FIG. **6**. The increased stress tolerance makes organisms that endogenously produce ascorbic acid particularly suitable for industrial production. Such organisms include plant and animal cells that produce ascorbic acid either naturally or through genetic engineering. (e.g., organisms described in Valpuesta, V. and Botella, M. A. (2004) Trends in Plant science 9, 573-577 and genetically engineered plant and animal cells.)

[0051] Organisms with increased stress tolerance that are to be used for industrial production may be created by any methods known to those of skill in the art for engineering recombinant organisms. The organism may be co-transformed with the necessary coding regions for production of L-ascorbic acid (i.e., ME, LGDH, ALO+/−MIP) and the coding sequences for the industrial product that the organism will produce. The organism may first be engineered to express the L-ascorbic acid coding sequences and then subsequently be transformed with coding regions for the industrial product. Alternatively, the organism may first be engineered to produce the industrial product and subsequently be transformed with the coding regions for production of L-ascorbic acid.

[0052] Endogenous production of L-ascorbic acid by the recombinant organism is particularly useful if the recombinant organism is cultivated under conditions of osmotic, pH, temperature, or oxidative stress. Osmotic stress is a condition in which the organism or cell encounters a difference in osmolarity from the optimal osmolarity defined for the respective microorganism. For example, in the yeast *S. cerevisiae*, an osmolarity greater than 500 mOsmol leads to a stress response.

[0053] A pH stress occurs if an organism or strain of organism encounters a difference in pH value from the optimal pH value for that strain of more than one to three pH units. For example, in the wild type strain of the yeast *S. cerevisiae*, the typical optimal pH for performance of bioprocesses is 5.0. A pH of less than 4.0 or more than 6.0 may cause a stress response in this strain that can affect the transcription of pH sensitive genes.

[0054] Temperature stress is a condition in which the organism encounters a cultivation temperature different the optimal temperature value for growth or production for a particular organism. In the yeast, *S. cerevisiae*, a temperature at or above 32° C. can cause stress responses. For the bacterium *E. coli*, a temperature at or above 38° C. can lead to stress responses.

[0055] Oxidative stress is a general term used to describe the steady state level of oxidative damage in a cell, caused by the reactive oxygen species (ROS). This damage can affect a specific molecule or the entire organism. Reactive oxygen species, such as free radicals and peroxides, represent a class of molecules that are derived from the metabolism of oxygen and exist inherently in all aerobic organisms. Oxidative stress results from an imbalance between formation and neutralization of pro-oxidants. Animal cells, as well as single-celled organisms, can be exposed to significant oxidative stress during standard cell culture conditions.

[0056] Endogenous production of L-ascorbic acid is also particularly useful in a cell or organism if it is subjected to stress due to overproduction of a metabolite or a protein. Such stresses may be indicated, for example, by the upregulation of genes related to the UPR (unfolded protein response), which is known in the art. (Foti, D. M., et al. (1999) J. Biol. Chem. 274, 30402-30409).

[0057] In one embodiment, the recombinant organism may be a yeast that has been engineered to produce and secrete lactic acid. The applications of lactic acid and its derivatives encompass many fields of industrial activities (e.g., chemistry, cosmetics, and pharmacy), as well as important aspects of food manufacture and use. Furthermore, today there is growing interest in the production of such an organic acid to be used directly for the synthesis of biodegradable polymer materials.

[0058] Lactic acid may be produced by chemical synthesis or by fermentation of carbohydrates using single-celled organisms. The latter method is now commercially preferred because organisms have been developed that produce exclusively one isomer, as opposed to the racemic mixture generated by chemical synthesis. The most important non-recombinant industrial organisms currently used to produce lactic acid, such as species of the genera *Lactobacillus, Bacillus*, and *Rhizopus*, produce L(+)-lactic acid. Production by fermentation of D(−)-lactic acid or mixtures of L(+)- and D(−)-lactic acid are also known.

[0059] During a typical lactic acid fermentation, the accumulation of lactic acid in the medium is detrimental to

metabolic activity. In addition, the accumulation of lactic acid lowers the pH of the medium, which also inhibits cell growth and metabolic activity. As a result, the extent of lactic acid production is reduced as the lactic acid product accumulates.

[0060] Methods for the construction of recombinant yeasts expressing at least one copy of a lactate dehydrogenase (LDH) gene, which shifts the glycolytic flux towards the production of lactic acid, have been described in U.S. Pat. Nos. 6,429,006 and 7,049,108, both of which are incorporated herein by reference. These references report that lactic acid can be produced by metabolically modified yeasts belonging to the genera of *Kluyveromyces, Saccharomyces, Torulaspora* and *Zygosaccharomyces*. While any yeast species could be used, these species are preferred because these strains can grow and/or metabolize at very low pH, especially in the range of pH 4.5 or less. In addition, genetic engineering methods for these strains are well-developed, and these strains are widely accepted for use in food-related applications.

[0061] The yield of lactic acid can be increased by increasing copy numbers of the LDH gene in each yeast. Higher yields (>80% g/g) of lactic acid may be obtained from these engineered yeast strains if both the ethanolic fermentation pathway and the use of pyruvate by mitochondria are replaced by lactic fermentation. The recombinant yeast can also be transformed to overexpress a lactate transporter, for example, the JEN1 gene encoding for the lactate transporter of *S. cerevisiae*, can to ensure secretion of the product.

[0062] The expression of a LDH gene in yeast strains allows the production of lactic acid at acid pH values so that the free acid is directly obtained and the cumbersome conversion and recovery of lactate salts are minimized. In this invention, the pH of the fermentation medium may initially be higher than 4.5, but will decrease to a pH of 4.5 or less, preferably to a pH of 3 or less at the termination of the fermentation.

[0063] The gene coding for LDH may be from any species (e.g., mammalian, such as bovine, or bacterial), and it may code for the L(+)-LDH or the D(−)-LDH. Alternatively, both types of LDH genes may be expressed simultaneously. In addition, any natural or synthetic variants of LDH DNA sequences, any DNA sequence with high identity to a wild-type LDH gene, any DNA sequence complementing the normal LDH activity may be used.

[0064] The co-expression of ascorbic acid in a lactic acid producing microorganism to improve the stress tolerance and robustness of that organism could be accomplished by introduction of ME, LGDH, ALO, and, optionally, MIP. The transformation of the yeast strains could be carried out by means of either integrative or replicative plasmid or linear vectors. In a particular embodiment of the invention, the recombinant DNA is part of an expression plasmid which can be of autonomous or integrative replication.

[0065] For the production of lactic acid, the recombinant yeast strains that endogenously produce ascorbic acid and produce and secrete lactic acid would be cultured in a medium containing a carbon source, D-glucose, and other essential nutrients. The lactic acid would be recovered at a pH of 7 or less, preferably at a pH of 4.5 or less, and even more preferably at a pH of 3 or less. Because the pH of the culture medium would be reduced, less neutralizing agent would be required. The formation of lactate salt would be correspondingly reduced and proportionally less regeneration of free acid would be necessary in order to recover lactic acid.

[0066] Because the recombinant yeast are more stress tolerant due to the endogenous production of L-ascorbic acid, the yeast cells separated from the lactic acid product could be utilized again as seed microorganisms for a fresh lactic acid fermentation. In addition, the yeast cells could be continuously separated and recovered during the lactic acid fermentation, and hence, the fermentation could be carried out continuously at low pH with less severe effects of pH and oxidative stress on yeast viability, production, productivity, and yield.

[0067] The following definitions are provided in order to aid those skilled in the art in understanding the detailed description of the present invention.

[0068] "Ascorbic acid" as well as "ascorbate" as used herein, refers to L-ascorbic acid.

[0069] "Ascorbic acid precursor" is a compound that can be converted by an organism of the present invention, either directly or through one or more intermediates, into L-ascorbic acid.

[0070] "Amplification" refers to increasing the number of copies of a desired nucleic acid molecule or to increase the activity of an enzyme, by whatsoever means.

[0071] "Codon" refers to a sequence of three nucleotides that specify a particular amino acid.

[0072] "DNA ligase" refers to an enzyme that covalently joins two pieces of double-stranded DNA.

[0073] "Electroporation" refers to a method of introducing foreign DNA into cells that uses a brief, high voltage DC charge to permeabilize the host cells, causing them to take up extra-chromosomal DNA.

[0074] "Endonuclease" refers to an enzyme that hydrolyzes double stranded DNA at internal locations.

[0075] "Engineered for industrial production" refers to a recombinant organism that has been genetically modified to produce an industrial product.

[0076] Enzyme 1.1.3.37, D-arabinono-1,4-lactone oxidase, refers to a protein that catalyzes the conversion of D-arabinono-1,4-lactone+O_2 to D-erythroascorbate+H_2O_2. The same enzyme due to broadness of substrate range catalyses the conversion of L-galactono-1,4-lactone+O_2 to L-ascorbic acid+H_2O_2. Erroneously the same enzyme is referred to as L-galactono-1,4-lactone oxidase (enzyme 1.1.3.24) (Huh, W. K. et al. (1998), Mol. Microbiol. 30, 895-903)

[0077] Enzyme 1.3.2.3, L-galactono-1,4-lactone dehydrogenase, refers to a protein that catalyzes the conversion of L-galactono-1,4-lactone+2 ferricytochrome C to L-ascorbic acid+2 ferrocytochrome C.

[0078] Enzyme 1.1.3.8, L-gulono-1,4-lactone oxidase, refers to a protein that catalyzes the oxidation of L-gulono-1,4-lactone to L-xylo-hexulonolactone which spontaneously isomerizes to L-ascorbic acid.

[0079] Enzyme GDP-mannose-3,5-epimerase (5.1.3.18), refers to a protein that catalyzes the conversion of GDP-mannose to GDP-L-galactose.

[0080] Enzyme myoinositol phosphatase (3.1.3.23), refers to a protein that catalyzes the conversion of L-galactose-1P to L-galactose. L-galactose-1-phosphatase has been annotated as inositol/myo-inositol monophosphatase galactose-1-phosphatase and may be referred to as MIP/VTC4 (Conklin, P. L. (2006) J. Biol. Chem. 281, 15662-70).

[0081] Other enzymes of interest, and their classification numbers, are as follows:

GDP-Mannose 3,5-epimerase	5.1.3.18
L-Galactono-1,4-lactone dehydrogenase	1.3.2.3
UDP-Glucuronate 4-epimerase	5.1.3.6
L-Gulono-1,4-lactone oxidase	1.1.3.8
Myoinositol 1-P monophosphatase	3.1.3.25
UDP-Glucose 4-epimerase	5.1.3.2
D-arabinose 1-dehydrogenase (NAD)	1.1.1.116
D-arabinose 1-dehydrogenase (NADP)	1.1.1.117

[0082] The term "expression" refers to the transcription of a gene to produce the corresponding mRNA and translation of this mRNA to produce the corresponding gene product, i.e., a peptide, polypeptide, or protein.

[0083] The term "fermentation" refers to a process in which organisms growing in a liquid or solid medium produce an industrial product. As used herein, the term does not refer exclusively to non-oxidative metabolism.

[0084] The phrase "functionally linked" or "operably linked" refers to a promoter or promoter region and a coding or structural sequence in such an orientation and distance that transcription of the coding or structural sequence may be directed by the promoter or promoter region.

[0085] The phrase "functionally transformed" refers to an organism that has been transformed with an exogenous nucleic acid and is capable of producing a functional protein or peptide encoded by that amino acid.

[0086] The term "gene" refers to chromosomal DNA, plasmid DNA, cDNA, synthetic DNA, or other DNA that encodes a peptide, polypeptide, protein, or RNA molecule, and regions flanking the coding sequence involved in the regulation of expression.

[0087] The term "genome" encompasses both the chromosomes and plasmids within a host cell. Encoding DNAs of the present invention introduced into host cells can therefore be either chromosomally integrated or plasmid-localized.

[0088] "Heterologous DNA" refers to DNA from a source different than that of the recipient cell.

[0089] "Homologous DNA" refers to DNA from the same source as that of the recipient cell.

[0090] "Hybridization" refers to the ability of a strand of nucleic acid to join with a complementary strand via base pairing. Hybridization occurs when complementary sequences in the two nucleic acid strands bind to one another.

[0091] The term "medium" refers to the chemical environment of the organism, comprising any component required for the growth of the organism and one or more precursors for the production of ascorbic acid. Components for growth and precursors for the production of ascorbic acid may or may be not identical.

[0092] "Open reading frame (ORF)" refers to a region of DNA or RNA encoding a peptide, polypeptide, or protein.

[0093] "Plasmid" refers to an extra chromosomal, replicatable piece of DNA.

[0094] "Polymerase chain reaction (PCR)" refers to an enzymatic technique to create multiple copies of one sequence of nucleic acid. Copies of DNA sequence are prepared by shuttling a DNA polymerase between two amplimers. The basis of this amplification method is multiple cycles of temperature changes to denature, then re-anneal amplimers, followed by extension to synthesize new DNA strands in the region located between the flanking amplimers.

[0095] The term "promoter" or "promoter region" refers to a DNA sequence, usually found upstream (5') to a coding sequence, that controls expression of the coding sequence by controlling production of messenger RNA (mRNA) or other functional RNAs, (e.g., tRNAs, rRNAs, sRNAs), by providing the recognition site for RNA polymerase and/or other factors necessary for start of transcription at the correct site.

[0096] A "recombinant cell" or "transformed cell" is a cell that contains a nucleic acid sequence not naturally occurring in the cell or an additional copy or copies of an endogenous nucleic acid sequence, wherein the nucleic acid sequence is introduced into the cell or an ancestor thereof by human action.

[0097] The term "recombinant vector" or "recombinant DNA or RNA construct" refers to any agent such as a plasmid, cosmid, virus, autonomously replicating sequence, phage, or linear or circular single-stranded or double-stranded DNA or RNA nucleotide sequence, derived from any source, capable of genomic integration or autonomous replication, comprising a nucleic acid molecule in which one or more sequences have been linked in a functionally operative manner. Such recombinant constructs or vectors are capable of introducing a 5' regulatory sequence or promoter region and a DNA sequence for a selected gene product into a cell in such a manner that the DNA sequence is transcribed into a functional mRNA, which may or may not be translated and therefore expressed.

[0098] "Restriction enzyme" refers to an enzyme that recognizes a specific sequence of nucleotides in double stranded DNA and cleaves both strands; also called a restriction endonuclease. Cleavage typically occurs within the restriction site or close to it.

[0099] "Selectable marker" refers to a nucleic acid sequence whose expression confers a phenotype facilitating identification of cells containing the nucleic acid sequence. Selectable markers include those, which confer resistance to toxic chemicals (e.g. ampicillin, kanamycin) or complement a nutritional deficiency (e.g. uracil, histidine, leucine).

[0100] "Screenable marker" refers to a nucleic acid sequence whose expression imparts a visually distinguishing characteristic (e.g. color changes, fluorescence).

[0101] "Transcription" refers to the process of producing an RNA copy from a DNA template.

[0102] "Transformation" refers to a process of introducing an exogenous nucleic acid sequence (e.g., a vector, plasmid, or recombinant nucleic acid molecule) into a cell in which that exogenous nucleic acid is incorporated into a chromosome or is capable of autonomous replication. A cell that has undergone transformation, or a descendant of such a cell, is "transformed" or "recombinant."

[0103] "Translation" refers to the production of protein from messenger RNA.

[0104] "Unit" of enzyme refers to the enzymatic activity and indicates the amount of micromoles of substrate converted per mg of total cell proteins per minute.

[0105] "Vector" refers to a DNA or RNA molecule (such as a plasmid, cosmid, bacteriophage, yeast artificial chromosome, or virus, among others) that carries nucleic acid sequences into a host cell. The vector or a portion of it can be inserted into the genome of the host cell.

[0106] The term "yield" refers to the amount of industrial product or L-ascorbic acid produced by the recombinant organism, as (molar or weight/volume) divided by the amount of precursor consumed (molar or weight/volume) multiplied by 100.

[0107] List of Abbreviations:

Asc L-ascorbic acid (vitamin C)

AGD L-galactono-1,4-lactone dehydrogenase (without signaling peptide)

ALO D-arabinono-1,4-lactone oxidase

ARA D-arabinose dehydrogenase

Gal L-galactono-1,4-lactone

Gul L-gulono-1,4-lactone

LGDH L-galactose dehydrogenase

ME Mannose epimerase

MIP Myoinositol phosphatase

RGLO L-gulono-1,4-lactone oxidase

TCA trichloroacetic acid

TPI triosephosphateisomerase

EXAMPLES

[0108] The following examples are included to demonstrate particular embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Materials and Methods

[0109] 1. Determination of Ascorbic Acid

[0110] Ascorbic acid was determined spectrophotometrically following the method of Sullivan, M. X. et al. (1955), Assoc. Off. Agr. Chem., 38, 514-518). The sample (135 μl) was mixed in a cuvette with 40 μl of H_3PO_4 (85%). Then 675 μl of α,α'-Bipyridyl (0.5%) and 135 μl $FeCl_3$ (1%) were added. After 10 min the absorbance at 525 nm was measured. In some experiments, the identity of the ascorbic acid was confirmed by HPLC (Tracer Extrasil Column C8, 5 μM, 15×0.46 cm, Teknokroma, S. Coop. C. Ltda. # TR-016077; Eluent: 5 mM cetyltrimethylammonium bromide, 50 mM KH_2PO_4 in 95/5H_2O/Acetonitrile; Flow rate: 1 ml min^{-1}, Detection UV @ 254 nm) with pure L-ascorbic acid (Aldrich, A9,290-2) as standard.

[0111] 2. Amplification of Specific Gene Sequences

[0112] To amplify specific gene sequences, PfuTurbo DNA polymerase (Stratagene #600252) was used on a GeneAmp PCR System 9700 (PE Appl. Biosystems, Inc.). Standard conditions used were: 400 μM dNTP, 0.5 μM primers, 0.5 mM $MgCl_2$ (in addition to the buffer), and 3.75 U Pfu per 100 μl reaction.

[0113] The sequences of the genes used have been publicly reported via Genbank, as follows, except for MIP. The MIP sequence listed as SEQ ID NO:4 differed from the Genbank sequence, accession no. NM_111155, by two translationally silent point substitutions: at bp271, A (NM_111155) to T (SEQ ID NO:4); at bp 685, T (NM_11155) to G (SEQ ID NO:4).

Gene	Genbank accession no(s).	SEQ ID NO:
ME	AY116953	3
MIP	n.a.	4
ALO	U40390, AB009401	5, 6
LGDH		7

[0114] The following program was used for amplification of ALO:

94° C.	5 min		
94° C.	45 s	}	33 cycles
50° C.	30 s		
72° C.	1 min 40 s		
72° C.	7 min		
4° C.	To completion		

[0115] The following program was used for amplification of LGDH:

94° C.	5 min		
94° C.	45 s	}	33 cycles
56° C.	30 s		
72° C.	1 min 40 s		
72° C.	7 min		

-continued

4° C.	To completion	

[0116] The following program was used for amplification of ME:

94° C.	5 min	
94° C.	15 s	} 30 cycles
50° C.	30 s	
72° C.	1 min 30 s	
72° C.	7 min	
4° C.	To completion	

[0117] The following program was used for amplification of MIP:

94° C.	5 min	
94° C.	15 s	} 28 cycles
59.8° C.	30 s	
72° C.	45 s	
72° C.	7 min	
4° C.	To completion	

[0118] Template DNA for LGDH, ME, and MIP: 50 ng plasmid cDNA library pFL61 *Arabidopsis* (ATCC #77500 (Minet M. et al. (1992), Plant J. 2, 417-422)). Template DNA for ALO: 50 ng genomic DNA from *S. cerevisiae* GRF18U, extracted using a standard method. PCR products were blunt-end cloned into the EcoRV site of pSTBlue-1 using the perfectly blunt cloning kit from Novagen Inc. (#70191-4).

Oligonucleotides used		Gene amplified
SEQ ID NO:8:	tttcaccatatgtctactatcc	ALO
SEQ ID NO:9:	aaggatcctagtcggacaactc	(yeast)
SEQ ID NO:10:	atgacgaaaatagagcttcgagc	LGDH
SEQ ID NO:11:	ttagttctgatggattccacttgg	(plant)
SEQ ID NO:12:	gcgccatgggaactaccaatggaaca	ME
SEQ ID NO:13:	gcgctcgagtcactcttttccatca	(plant)
SEQ ID NO:14:	atccatggcggacaatgattctc	MIP
SEQ ID NO:15:	aatcatgcccctgtaagccgc	(plant)

[0119] 3. Plasmid Construction

[0120] The naming convention used herein is that pSTBlue-1 containing, for example, ALO in the sense direction regarding its multiple cloning site (MCS) was designated pSTB ALO-1. In a further example, pSTBlue-1 containing ALO in the antisense direction regarding its MCS was designated pSTB ALO-2, and so on.

[0121] Inserts were cloned using either the pYX series (R&D Systems, Inc.) or the centromeric expression plasmids pZ_3 and pZ_4 (P. Branduardi, et al. The Yeast *Zygosaccharomyces bailii*: a New Host for Heterologous Protein Production, Secretion and for Metabolic Engineering Applications, FEBS Yeast Research, FEMS Yeast Res. (2004) 4, 493-504). Standard procedures were employed for all cloning purposes, (Sambrook, J. and Russell, D. W., *Molecular Cloning: A Laboratory Manual,* 3rd Edition, Cold Spring Harbor Laboratory Press, 2001).

pSTB LGDH-1	EcoRI	pYX022	pH LGDH	HIS 3 (marker)
pSTB ALO-1	EcoRI	pYX042	pL ALO	LEU 2 (marker)
pSTB ME-1	EcoRI	pZ_3	pZ_3 ME	Kan^r (marker)
pSTB ME-1	EcoRI	pZ_4	PZ_4 ME	Hph^r (marker)
pSTB MIP-1	EcoRI	pYX012	pU MIP	URA 3 (marker)

[0122] For all the work performed below, the yeast control strains were transformed with the corresponding empty vectors.

[0123] 4. Yeast Cultivation and Examination:

[0124] Yeast strains used were *S. cerevisiae* GRF18U (Brambilla, L. et al., 1999, FEMS Microb. Lett. 171, 133-140), *S. cerevisiae* GRFc (Brambilla et al. 1999 FEMS Microb. Lett. 171: 133-140), *S. cerevisiae* BY4742 (MATα; his3; leu2, lys2; ura3, EuroScarf Accession No. Y10000), *S. cerevisiae* YML007w (BY4742; MATα; his3; leu2, lys2; ura; YML007w::KanMX4 (the yap1 deleted strain) EuroScarf Accession No. Y10569); CEN.PK 113-5D (MATα ura3-52; cir+) (see, for example, VanDijken et al. (2000) Enzyme Microb. Technol. 26, 706-714); and BY4741 (MATα; his3; leu2; met 15; ura3, Euroscarf Accession No. Y00000), or strains derived from them through transformation with the different developed plasmids. All strains were cultivated in shake flasks in minimal medium (0.67% w/v YNB (Difco Laboratories, Detroit, Mich. #919-15), 2% w/v glucose or mannose, with addition of the appropriate amino acids or adenine or uracil, respectively, to 50 mL^{-1}) and/or the appropriate antibiotic (G418 or hygromicin to 500 mg/l and 400 mg/l, respectively) under standard conditions (shaking at 30° C.). The initial optical density at 660 nm was about 0.05 for ascorbic acid determination, and 0.1 for the kinetics of the recovery from oxidative stress.

[0125] Cells were recovered by centrifugation at 4000 rpm for 5 min at 4° C., washed once with cold distilled H_2O, and treated as follows: for determination of intracellular ascorbic acid, cells were resuspended in about 3 times the pellet volume of cold 10% TCA, vortexed vigorously, kept on ice for about 20 min, and then the supernatant was cleared from the cell debris by centrifugation.

[0126] 5. Yeast Transformation:

[0127] Transformation of yeast cells was performed by the standard LiAc/ss-DNA/PEG method (Gietz, R. D. and Schiestl, R. H. (1996), Transforming Yeast with DNA, Methods in Mol. and Cell. Biol.).

[0128] Experimental Results

[0129] 6. Expression of *Arabidopsis thaliana* ME, MIP, LDGH and *S. cerevisiae* ALO in GRF18U

[0130] The genes encoding *A. thaliana* ME, *S. cerevisiae* ALO, *A. thaliana* LGDH, and *A. thaliana* MIP were placed

under the control of the TPI (triosephosphateisomerase) promoter each on its own integrative plasmid, except ME, which was sub-cloned in a centromeric plasmid. Two or more of the genes were integrated into *S. cerevisiae* GRF18U and BY4742. Each gene was integrated at a unique locus.

[0131] FIG. **1** provides a schematic representation of the current understanding of the physiological biosynthetic pathway leading from D-glucose to L-ascorbic acid in plants. The following enzymes are involved: A, L-galactono-1,4-lactone dehydrogenase (1.3.2.3), B, L-galactose dehydrogenase, C, myoinositol phosphatase (3.1.3.23), D, pyrophosporylase, E, GDP-mannose-3,5-epimerase (5.1.3.18), F, mannose-1-phosphate guanylyltransferase (2.7.7.22), G, phosphomannomutase (5.4.2.8), H, mannose-6-phosphate isomerase (5.3.1.8), I, glucose-6-phosphate isomerase (5.3.1.9), J; hexokinase (2.7.1.1).

[0132] In the pathway shown in FIG. **1**, ALO catalyzes reaction A, LGDH catalyzes reaction B, ME catalyzes reaction E, and MIP catalyzes reaction C.

[0133] Wild-type yeast cells are known to produce GDP-mannose (reactions F-J in FIG. **1**) and to transport it to the endoplasmic reticulum.

[0134] The table below shows the conversion of D-Glucose and D-Mannose to ascorbic acid by *S. cerevisiae* GRFc (control), or *S. cerevisiae* GRF18U transformed with (i) ALO and LDGH; (ii) ALO, LDGH and ME; or (iii) ALO, LDGH, ME and MIP. Cells were grown on mineral medium (2% glucose or mannose, 0.67% YNB) starting from an OD^{660} of 0.05. After 24 hours of growth, ascorbic acid was determined. While both the wild-type GRFc and GRF18U cells transformed with ALO and LGDH did not accumulate ascorbic acid, cells transformed with ALO, LDGH and ME, or ALO, LDGH, ME and MIP, respectively unexpectedly accumulated considerable amounts (i.e. greater than background levels) of ascorbic acid.

[0135] Transformed yeast were batch grown on glucose- or mannose-based media:

Expressed gene	Total (ascorbate plus erythroascorbate) on glucose-containing media	Total (ascorbate plus erythroascorbate) on mannose-containing media
Wt (control)	0.0205	0.0220
ALO, LGDH (control)	0.0210	0.0221
ALO, LDGH, ME	0.0302	0.0332
ALO, LDGH, ME, MIP	0.0450	0.0296

(Total (ascorbate plus erythroascorbate) values are mg/OD^{660} of Biomass/L)

[0136] The values determined in the control strain indicate the production of erythroascorbate normally produced by wild type yeasts.

[0137] We conclude that the yeast endogenously possesses activities which can nonspecifically catalyze reactions from GDP-L-galactose to L-galactose (see FIG. **1**). Specifically, though not to be bound by theory, we conclude that GDP-L-galactose spontaneously hydrolyses to L-galactose-1-P and that a nonspecific phosphatase catalyzed the conversion of L-galactose-1-P to L-galactose, which was then converted to L-ascorbic acid by LGDH and ALO. MIP provided superior catalysis of L-galactose-1-P to L-galactose than did the putative nonspecific phosphatase (ALO, LGDH, ME, MIP vs. ALO, LGDH, ME).

[0138] We did not observe any ascorbic acid accumulation in the medium.

[0139] 7. Sensitivity to Oxidative Stress

[0140] FIG. **2** shows that YML007w yeast hosts are particularly sensitive to oxidative stress. Yap1p activates genes required for the response to oxidative stress; deletion of this gene leads to the observed phenotype (Rodrigues-Pousada C A, et al. (2004) FEBS Lett.

[0141] 567, 80-85)

[0142] The following yeast strains have been analyzed:

[0143] BY4742 (▲).

[0144] YML007w (○)

[0145] FIG. **2**A. The yeast strains were grown on mineral medium (2% glucose, 0.67% YNB) starting from an OD^{660} of 0.1.

[0146] FIG. **2**B. The yeast strains were grown on mineral medium (2% glucose, 0.67% YNB) starting from an OD^{660} of 0.1 in the presence of 0.8 mM of H_2O_2.

[0147] FIG. **2**C. The yeast strains were grown on mineral medium (2% glucose, 0.67% YNB) starting from an OD^{660} of 0.1 in the presence of 1.0 mM of H_2O_2.

[0148] The two strains grew in the absence of H_2O_2 (FIG. **2**A) while growth of the YML007w yeast host was strongly delayed in medium containing 0.8 mM of hydrogen peroxide (FIG. **2**B) and completely impaired in the medium containing 1 mM of hydrogen peroxide (FIG. **2**C).

[0149] 8. Effect of Ascorbic Acid in Media on Stress Tolerance

[0150] FIG. **3** shows that the growth sensitivity of YML007w yeast, as shown in FIG. **2**, can be rescued by adding ascorbic acid to the medium, and that the effect of ascorbic acid in the medium on robustness is concentration dependent and can be optimized for different yeast strains.

[0151] FIG. **3**A. The yeast strains were grown on minimal medium (2% glucose, 0.67% YNB) starting from an OD^{660} of 0.1 in presence of 0.8 mM of H_2O_2. Ascorbic acid was added at T=0 at a final concentration of 15 mg/L. BY4742 (▲); YML007w (○).

[0152] FIG. **3**B. The yeast strains were grown on mineral medium (2% glucose, 0.67% YNB) starting from an OD^{660} of 0.1 in presence of 1.0 mM of H_2O_2. Ascorbic acid was added at T=0 at a final concentration of 15 mg/L. BY4742 (▲); YML007w (○).

[0153] FIG. **3**C. Three yeast strains (GRFc, BY4741, and CEN.PK 113-5D) were grown in 2×YNB medium (2% glucose, 1.34% YNB), containing lactic acid at 40 g/l, pH3. Ascorbic acid was added to the medium at the concentrations shown. The data demonstrate that the negative effects of lactic acid on growth can be overcome by exogenous ascorbic acid, and that the effect of ascorbic acid is dose dependent.

[0154] 9. Effect of Endogenous Ascorbic Acid on Sensitivity to Oxidative Stress

[0155] FIG. **4** shows that the growth defects of the YML007w yeast hosts can be rescued following expression of ALO, LDGH, ME, and MIP.

[0156] The following yeast strains have been analyzed:

[0157] BY4742 (▲)

[0158] YML007w expressing ALO, LDGH and ME (□)

[0159] YML007w expressing ALO, LDGH, ME and MIP (■)

[0160] FIG. **4**A. The yeast strains were grown on minimal medium (2% glucose, 0.67% YNB) starting from an OD^{660} of 0.1 in presence of 0.8 mM of H_2O_2.

[0161] FIG. **4**B. The yeast strains were grown on minimal medium (2% glucose, 0.67% YNB) starting from an OD^{660} of 0.1 in presence of 1.0 mM of H_2O_2.

[0162] Endogenous production of ascorbic acid "rescued" the yeast from stress-induced growth inhibition in a manner similar to that obtained by adding ascorbic acid to the culture medium (see FIG. **3**).

[0163] 10. Effect of Endogenous Ascorbic Acid on Robustness of GRF Yeast Strains

[0164] FIG. **5** shows that the wild type GRF yeast strain is sensitive to fermentative stress conditions (stress condition induced by adding 2 mM of H_2O_2); surprisingly, the recombinant yeast strains producing ascorbic acid show a strong robustness, indicating an increased tolerance to stress. The following yeast strains were analyzed: GRFc (closed triangle); GRF18U expressing ALO, LDGH and ME (open square); and GRF18U expressing ALO, LDGH, ME and MIP (closed square).

[0165] FIG. **5**A. The yeast strains were grown on mineral medium (2% glucose, 0.67% YNB) starting from an OD660 of 0.1.

[0166] FIG. **5**B. The yeast strains were grown on mineral medium (2% glucose, 0.67% YNB) starting from an OD660 of 0.1 in presence of 2.0 mM of H_2O_2. The wild type strain does not consume glucose.

[0167] All the strains used in this experiment bear the same auxotrophic complementation and the same antibiotic resistance cassettes (that are necessary for the expression of the different heterologous genes), so that it was possible to use the same media for all of them, either the ones expressing 3 or 4 heterologous genes or the wild type strain.

[0168] For this experiment, as a classical example of stress, we challenged wild type yeast cells with H_2O_2. As expected, wild type cells grow well in the absence of H_2O_2 (FIG. **5**A), but the same yeast cells do not grow in the presence of the H_2O_2 (FIG. **5**B). It is generally accepted that this external stressor leads to damage to DNA, damage to lipids, damage to proteins, and damage to membranes, among other subcellular structures, and ultimately leads to a loss of cell viability and cell integrity. Therefore, it is not surprising that the presence of this stressor leads to zero production, zero productivity and zero yield of the product (in this case, wild type yeast biomass), as shown in FIG. **5**B.

[0169] By the transformation of wild type GRF yeast with (i) LGDH, ALO, and ME or (ii) LGDH, ALO, ME and MIP, the recombinant yeast produced ascorbic acid, as described above, whereas wild type yeasts do not naturally produce ascorbic acid. Surprisingly, the bioprocess based on these recombinant yeasts showed a high production, high productivity, and a high yield of the product, yeast biomass (FIG. **5**B). Values for production, productivity, and yield are greater than 0.00 in the recombinant yeast (values for the control strain).

[0170] This experiment shows the two recombinant GRF yeast strains are more tolerant to stress than wild type GRF yeast, and may therefore be more suitable for certain industrial processes. Though not to be bound by a single theory, we consider it likely the recombinant yeast are less sensitive to diverse stressors, possibly through both direct scavenging of reactive oxygen species (ROS) by ascorbic acid and interference by ascorbic acid with unwanted stress reactions, such as apoptosis, cell death, viability loss, and loss of cell integrity.

[0171] 11. Effect of Endogenous Ascorbic Acid on ROS and Viability

[0172] The *S. cerevisiae* strains YML007w and YML007w transformed to express ALO, LDGH, ME, and MIP were grown in minimal glucose medium with or without addition of H_2O_2. Each culture was then split into two, and one was stained with dehydrorodamine for the detection of reactive oxygen species (ROS), the other was stained with propidium iodide for viability determination. Samples were then analyzed with a flow cytometer and compared. FIG. **6** demonstrates a correlation between ascorbic acid production and reduction in ROS formation, as well as reduction of the fraction of nonviable cells.

[0173] 12. Effect of Endogenous Ascorbic Acid on Sensitivity to Low pH and Lactic Acid

[0174] The *S. cerevisiae* strains BY4742c and BY4742 transformed to express ALO, LDGH, ME, MIP were inoculated in minimal glucose medium, minimal glucose medium at low pH (2.2), or minimal glucose medium at pH 3.0 containing 38 g/l of lactic acid. FIG. **7** shows growth curves for BY4742c (open squares) and the same yeast background transformed to produce ascorbic acid (dark squares) in minimal glucose medium, pH 2.2 (FIG. **7***a*), and in minimal glucose medium containing 38 g/l lactic acid, pH 3.0 (FIG. **7***b*). In the transformed yeast strain producing ascorbic acid, peak levels of cells at low pH are approximately three-fold greater and peak levels of cells in medium containing lactic acid are approximately five-fold greater compared with the non-transformed strain.

[0175] The same experiment was conducted after the two yeast strains were grown for about 24 hours in minimal glucose medium and then inoculated in minimal glucose medium at low pH (2.2), or minimal glucose medium at pH 3.0 containing 38 g/l of lactic acid. The results are shown in FIG. **8**. At low pH, the transformed strain producing lactic acid showed more than a six-fold increase in peak cell numbers compared with the non-transformed strain (FIG. **8***a*). In medium containing lactic acid, the non-transformed strain showed no increase in growth, whereas the transformed yeast strain producing ascorbic acid showed exponentional growth with an approximately 3.5 fold increase at peak levels (FIG. **8***b*).

[0176] 13. Effect of Exogenous Ascorbic Acid on Growth of Lactic Acid Producing Yeast m850.

[0177] *S. cerevisiae* strain NRRL Y-30696 was inoculated in minimal glucose medium and 2.78 g/L $CaCO_3$ or minimal glucose medium with 2.78 g/L $CaCO_3$ and 0.16, 0.3, or 0.6 g/L ascorbic acid. OD660 (open symbols) and lactic acid (closed symbols) were monitored with time. The pH dropped in each case to 2.5 at 67 hours. FIG. **9** shows that growth, as measure by OD660, increased with increasing ascorbic acid, 0 g/L (O), 0.16 g/L (+), 0.3 g/L (▲), or 0.6 g/L (♦), while lactic acid production was equivalent at each level.

[0178] 14. Construction of a Yeast Strain Co-Producing Lactic Acid and Ascorbic Acid.

[0179] *S. cerevisiae* NRRL Y-30696 (Y-30696) has previously been engineered to produce lactic acid. The ability to co-produce a low level of endogenous ascorbic acid could be introduced by integrating the genes required for ascorbic acid production into Y-30696. As shown above, production of significant endogenous L-ascorbic acid can be achieved by the expression of sequences encoding ME, LGDH, and ALO+/−MIP. One or more of these genes, functionally coupled to an appropriate promoter, could be added to the L-LDH bearing plasmid of Y-30696, while additional genes, coupled to appropriate promoters, could be introduced at the sites of the deleted PDC genes. Methods for these steps are known in the art, and are found in Sauer, M., et al. (2004) Applied Environmental Microbiology 70, 6086-6091.

[0180] While the compositions and methods and yeast strains of this invention have been described in terms of particular embodiments, it will be apparent to those of skill in the art that variations may be applied without departing from the concept, spirit and scope of the invention.

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What is claimed is:

1. A method of increasing stress tolerance in a recombinant organism that is engineered for industrial production of at least one product comprising functionally transforming the recombinant organism with a coding region encoding a mannose epimerase (ME), a coding region encoding an L-galactose dehydrogenase (LGDH), and a coding region encoding a D-arabinono-1,4-lactone oxidase (ALO), whereby the recombinant organism is enabled to produce ascorbic acid endogenously.

2. The method of claim 1, wherein the recombinant organism is further functionally transformed with a coding region encoding a myoinositol phosphatase (MIP).

3. The method of claim 1, wherein the recombinant organism is further functionally transformed with a coding region encoding an enzyme selected from the group consisting of L-galactono-1,4-lactone dehydrogenase (AGD), D-arabinose dehydrogenase (ARA), and L-gulono-1,4-lactone oxidase (GLO).

4. The method of claim 1, wherein the recombinant organism produces lactic acid.

5. The method of claim 1, wherein the recombinant organism is an organism selected from the group consisting of bacteria, yeast, filamentous fungi, and animal cells.

6. The method of claim 1, wherein the recombinant organism is a yeast belonging to a genus selected from the group consisting of *Saccharomyces, Zygosaccharomyces, Candida, Hansenula, Kluyveromyces, Debaromyces, Nadsonia, Lipomyces, Torulopsis, Kloeckera, Pichia, Schizosaccharomyces, Trigonopsis, Brettanomyces, Cryptococcus, Trichosporon, Aureobasidium, Lipomyces, Phaffia, Rhodotorula, Yarrowia*, and *Schwanniomyces*.

7. The method of claim 5, wherein the recombinant organism is a yeast selected from the group consisting of *S. cerevisiae* strain GRF18U; *S. cerevisiae* strains W3031B, BY4741, BY4742, CEN.PK 113-5D and YML007w; *K. lactis* strain CBS2359; *Z. bailii* strain ATCC 60483; *S. cerevisiae* strains NRRL Y-30696, NRRL Y-30698, NRRL Y-30742; *K. lactis* strains PM6-7/pEPL2, PMI/C1[pELP2]; *Z. bailii* strains ATTC36947/pLAT-ADH, ATCC60483/pLAT-ADH.

8. The method of claim 5, wherein the recombinant organism is a bacterium of a genus selected from the group consisting of *Bacillus, Escherichia, Lactobacillus, Lactococcus, Pseudomonas*, and *Acetobacter*.

9. The method of claim 5, wherein the recombinant organism is a bacterium selected from the group of bacterial strains producing lactic acid consisting of *Bacillus coagulans, Lactobacillus helveticus, Lactobacillus delbrueckii, Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus pentosus*, and *Streptococcus thermophilus*.

10. The method of claim 5, wherein the recombinant organism is a filamentous fungus of a genus selected from the group consisting of *Aspergillis, Rhizopus*, and *Trichoderma*.

11. The method of claim 5, wherein the recombinant organism is a filamentous fungus selected from the group consisting of *Aspergillus kawachii, Aspergillus nidulans, Aspergillus niger, Aspergillus oryzae, Rhizopus arrhizus, Rhizopus microsporus, Rhizopus oryzae, Trichoderma harzianum, Trichoderma reesei*, and *Trichoderma viride*.

12. The method of claim 1, wherein the ME has at least about 95% identity with SEQ ID NO:1.

13. The method of claim 2, wherein the MIP has at least about 95% identity with SEQ ID NO:2.

14. The method of claim 1, wherein the recombinant organism is a yeast, and wherein the yeast is engineered to produce at least one product selected from the group consisting of organic acids, amino acids, vitamins, polyols, solvents, biofuels, therapeutics, vaccines, proteins, and peptides.

15. The method of claim 1, wherein the recombinant organism is a yeast, and wherein the yeast is engineered to produce organic acids.

16. The method of claim 1, wherein the recombinant organism is a yeast, and wherein the yeast is engineered to produce lactic acid.

17. The method of claim 1, wherein the recombinant organism is a bacterium and wherein the bacterium is engineered to produce at least one product selected from the group consisting of organic acids, amino acids, vitamins, polyols, solvents, biofuels, therapeutics, vaccines, proteins, and peptides.

18. The method of claim 1, wherein the recombinant organism is a bacterium and wherein the bacterium is engineered to produce organic acids.

19. The method of claim 5, wherein the recombinant organism is a bacterium and wherein the bacterium is engineered to produce lactic acid.

20. The method of claim 1, wherein the recombinant organism is a filamentous fungus and wherein the filamentous fungus is engineered to produce at least one product selected from the group consisting of citric acid, lactic acid, and enzymes.

21. A method of increasing stress tolerance in a recombinant organism that is engineered for industrial production of at least one product, comprising culturing the recombinant organism in a medium containing an effective amount of ascorbic acid.

22. The method of claim 21, wherein the effective amount of L-ascorbic acid is 0.005 to 2.0 grams/liter.

23. The method of claim 21 wherein the effective amount of L-ascorbic acid is 0.015 to 0.1 gram/liter.

24. The method of claim 21, wherein the recombinant organism is engineered for the industrial production of lactic acid.

25. The method of claim 21, wherein the recombinant organism is a bacterium, a yeast, a filamentous fungus, or an animal cell.

26. A method of increasing stress tolerance in an organism that produces lactic acid comprising culturing the organism in a medium containing 0.005 to 2.0 grams/liter of ascorbic acid.

* * * * *

Influence of Low Temperature on Productivity, Proteome and Protein Phosphorylation of CHO Cells

Hitto Kaufmann, Xenia Mazur, Martin Fussenegger, James E. Bailey

Institute of Biotechnology, ETH Zürich, CH-8093 Zürich, Switzerland; telephone: +41 1 633 31 70; fax +41 1 633 10 51

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Abstract: Proliferation of mammalian cells can be controlled by low cultivation temperature. However, depending on cell type and expression system, varying effects of a temperature shift on heterologous protein production have been reported. Here, we characterize growth behavior and productivity of the Chinese hamster ovary (CHO) cell line XM111-10 engineered to synthesize the model-product-secreted alkaline phosphatase (SEAP). Shift of cultivation temperature from 37°C to 30°C caused a growth arrest mainly in the G1 phase of the cell cycle concomitant with an up to 1.7-fold increase of specific productivity. A low temperature cultivation provided 3.4 times higher overall product yield compared to a standard cultivation at 37°C. The cellular and molecular mechanisms underlying the effects of low temperature on growth and productivity of mammalian cells are poorly understood. Separation of total protein extracts by two-dimensional gel electrophoresis showed altered expression levels of CHO-K1 proteins after decrease in cultivation temperature to 30°C. These changes in the proteome suggest that mammalian cells respond actively to low temperature by synthesizing specific cold-inducible proteins. In addition, we provide the first evidence that the cold response of mammalian cells includes changes in postranslational protein modifications. Two CHO proteins were found to be phosphorylated at tyrosine residues following downshift of cultivation temperature to 30°C. Elucidating cellular events during cold exposure is necessary for further optimization of host-cell lines and expression systems and can provide new strategies for metabolic engineering. *Biotechnol Bioeng* **63**: 573–582, 1999.

INTRODUCTION

All living cells show a distinct response to external stresses such as temperature, pressure, osmotic stress, and oxygen deprivation. The most common cellular response includes changes in gene expression resulting in the synthesis of specific sets of stress proteins. The effect of heat shock on prokaryotic and eukaryotic cells has been extensively studied (for reviews see Craig et al., 1985; Hightower and Hendershot, 1997). Heat-shock stress leads to transient induction of heat-shock proteins (HSPs), a group of proteins that is highly conserved in all organisms from bacteria to mammals, many of which act as molecular chaperones for protein folding.

Recent studies focusing on the response of cells to a decrease in temperature led to the discovery of cold-inducible proteins. However, unlike HSPs, cold-shock proteins are not conserved among all species (for a review see Thieringer et al., 1998). In *Escherichia coli*, a temperature shift from 37°C to 10°C leads to transient inhibition of most protein synthesis. Growth is suppressed for a fourth period during which cells adapt to resume growth (Jones et al., 1987). During this lag phase, strong induction of the bacterial cold-shock proteins occurs. These cold-shock proteins have diverse functions and include, for example, the ribosome-associated initiation factor IF-2 (Jones et al., 1987) and the recombination factor RecA (Walker et al., 1984). The cold-inducible bacterial proteins CspA, CspB, and CspG share the cold-shock domain (CSD) as a common motif and are thought to function as RNA/DNA chaperones, assisting translation and replication at low temperatures (Jiang et al., 1997; Thieringer et al., 1998). The CSD is also present in eukaryotic Y-box transcription factors; however, Y-box proteins do not demonstrate cold inducibility (Wolffe et al., 1994).

Eukaryotic organisms also exhibit changes in gene expression in response to low temperature. *Saccharomyces cerivisiae* cells shifted from 30°C to 10°C growth with a shorter doubling time after a 1-h-growth lag (Kondo et al., 1992). Four different proteins were found to be synthesized at elevated levels after downshift of temperature. For example, the level of the NSR1 protein increased three-fold upon cold shock. This protein acts as an auxiliary factor for ribosome biosynthesis at low temperatures (Kondo et al., 1992).

Furthermore, response to low temperature has been reported in plants and many plant genes were shown to be induced by cold stress (Shinozaki and Yamaguchi-Shinozaki, 1996; Jaglo-Ottosen et al., 1998; Baker et al., 1994; Thomashow, 1994). These changes in gene expression are triggered by at least five signal-transduction pathways in the plant cell (Shinozaki et al., 1996). A variety of

Correspondence to: James Bailey

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functions have been described for the products of cold-inducible plant genes, including RNA-binding and chaperone activity (Carpenter et al., 1994).

The effects of low temperature on physiology, growth, and gene expression in mammalian cells have not been extensively studied. A temperature shift from 37°C to 30–33°C prolongs the total generation time of cultured mammalian cells (Rao and Engelberg, 1965; Watanabe and Okada, 1967). Recent cloning of the murine cold-inducible RNA-binding protein CIRP provided first evidence for upregulation of a mammalian gene product in response to low temperature (Nishiyama et al., 1997). It is important to note that overexpression of CIRP in mouse cells at 37°C caused accumulation of cells in the G1 phase of the cell cycle (Nishiyama et al., 1997). Likewise, the transcript level of RBM3, another member of the glycine-rich RNA-binding protein family, was reported to be significantly higher in human cells after temperature shift to 32°C (Danno et al., 1997).

Mammalian cell lines are widely used as host-production-cell lines in bioprocesses to produce pharmaceutically important proteins such as blood-clotting factors, monoclonal antibodies, growth factors, cytokines, and trombolytics. Overall heterologous protein production, product quality, and cell viability are critical parameters in optimization of mammalian cell-culture-production processes. In this respect, low-temperature cultivation has been discussed as a step to improve batch-culture performance (Bloemkolk et al., 1992; Giard et al., 1982; Reuveny et al., 1993; Sureshkumar and Mutharasan, 1991; Weidemann et al., 1994). Although these reports agree that a decrease in cultivation temperature leads to prolonged culture viability, the effects of low temperature on heterologous protein production of mammalian cells varied among different studies (Bloemkolk et al., 1992; Furukawa and Ohsuye, 1998; Giard et al., 1982; Reuveny et al., 1986; Sureshkumar and Mutharasan, 1991; Weidemann et al., 1994). For example, cultivation of hybridoma cells at low temperatures resulted in the loss of specific monoclonal antibody productivity (Sureshkumar and Mutharasan, 1991) while CHO cells secreting human recombinant antithrombin-III (ATIII) did not demonstrate changes in specific cellular productivity after temperature downshift to 33°C (Roessler et al., 1996). In this case, a lengthened production phase resulted in higher ATIII product yields. In summary, lowering culture temperature generally suppresses cell growth but its effect on cellular productivity is variable among different cell lines and expression systems.

Temperature is a process parameter by which the proliferation rate in production processes can be controlled. Cold-induced growth arrest was found to be G1-phase specific in CHO-batch cultures (Moore et al., 1997). Previously, we demonstrated that controlling proliferation of mammalian cells by overexpression of the growth inhibitory protein $p27^{KIP}$ leads to higher specific production of secreted alkaline phosphatase (SEAP) (Fussenegger et al., 1997; 1998; Muzar et al., 1998). p27 is a member of the CIP/KIP family of cell-cycle inhibitors (CKIs). This protein acts as a negative regulator of cyclin-dependent kinases (CDKs), reaching its maximum-inhibitory activity in the G1-phase of the cell cycle (see Hengst and Reed, 1998 for a review). Several cell lines were constructed by stable transfection of CHO-K1 cells with a dicistronic-expression-plasmid encoding p27 and SEAP under the control of the cytomegalovirus-derived tetracycline-regulatable promoter $P_{hCMV*-1}$. Overexpression of p27 led to accumulation of cells in the G1-phase and growth-arrested cells exhibited enhanced SEAP production compared to proliferating cells.

In this study, we investigated the effect of low-cultivation temperature on productivity of recombinant CHO cells and molecular changes that accompany a temperature shift from 37°C to 30°C. We characterized growth behavior and productivity of the cell line XM111-10 at low temperature. This cell line was constructed by engineering CHO cells to synthesize SEAP under the control of the P_{CMV*-1}-based expression system. To acquire initial information on the molecular responses to low-temperature cultivation, protein patterns of CHO cells shifted to low temperature were determined by using two-dimensional gel electrophoresis and compared to the proteome at 37°C. Furthermore, assays were conducted to check if changes in tyrosine phosphorylation might play a role in the cold response of CHO cells.

MATERIALS AND METHODS

Cell Culture

Chinese hamster ovary cells (CHO-K1, ATCC:CCL 61) were cultured in FMX-8 medium (Dr. Messi, Cell Culture Systems, Zurich, Switzerland) supplemented with 10% fetal calf serum (FCS, Boehringer Mannheim, Lot Nr. 14713602) and 100 IU/mL penicillin at 37°C in a humidified atmosphere of 5% CO_2 in air. The cell line XM111-10 derives from the CHO-cell line XMK1-9 (Fussenegger et al., 1997; Mazur et al., submitted). XMK1-9 cells constitutively express the tetracycline-responsive transactivator (tTA) necessary for tetracycline-repressible gene expression (Gossen and Bujard, 1992). The pMF111 expression construct encoding the secreted alkaline phosphatase (SEAP) under the control of the tetracycline-repressible promoter $P_{hCMV*-1}$ was stably transfected into XMK1-9 cells to give the CHO-cell line XM111-10 (Fussenegger et al., 1997; Mazur et al., 1998). XM111-10 cells were cultured in 10% serum-enriched FMX-8 medium supplemented with 100 IU/mL penicillin, 400 μg/mL G418, and 6 μg/mL puromycin. The cells were cultivated in the absence of tetracycline to allow SEAP transcription at the maximum level. For temperature-shift experiments, cells were grown at 37°C until the culture reached exponential growth phase and were then shifted to 30°C in a humidified atmosphere of 5% CO_2 in air. After indicated times, cells were harvested for 2-D electrophoresis-sample preparation or to obtain whole cell extracts for Western blot analysis. The growth curve of XM111-10 cells

was determined by counting the cells in a 25 cm^2 cell culture flasks (T25) every 24 h. The cultivation was stopped after cells reached the stationary/death phase. For each day an initial cell population of 2×10^5 cells was seeded in triplicate into T25 flasks.

At 24-h time intervals, the cells were completely detached with cell dissolvation solution (SIGMA) and diluted 1:1000 with filter-purified Casyton buffer. The cell number was determined as mean values of six independent readings taken by a Casy 1 cell counter (Schaerfe System, Reutlingen, Germany).

SEAP and Metabolic Assays

Five hundred μL of culture supernatant were incubated for 5 min at 65°C to inactivate endogenous phosphatase activity. Cell debris were separated in a subsequent centrifugation step for 2 min at 10,000*g*. Four hundred μL of the supernatant and 500 μL 2 × SEAP buffer (20 m*M* *L*-homoarginine, 2*M* diethanolamine pH 9.8, 1 m*M* $MgCl_2$) were transferred to a half-micro cuvette. The enzymatic reaction was initiated by adding 100 μL of 120 m*M* *p*-nitrophenylphosphate (SIGMA 104 phosphatase substrate). The increase in light absorbance at 405 nm which accompanies the hydrolysis of *p*-NPP was measured at appropriate time intervals, and is a direct proportional measure for SEAP concentration (Berger et al., 1988). One mU is defined as the amount of SEAP which will hydrolyze 1.0 m*M* of *p*-NPP per min, and this equals an increase of 0.04 A_{405} units per min.

To assess the metabolic activities of XM111-10 cells in low temperature vs. 37°C cultivations, we measured the specific glucose uptake of cultures under both conditions. Initial populations of 2.5×10^5 cells were seeded into T75 flasks, and glucose concentrations were measured in the culture supernatants using an automated enzyme analysis system according to the manufacturer's protocol (Synchron CX5CE, Fullerton, CA, Beckman).

Flow Cytometric Analysis

To quantify changes in cell-cycle distribution after temperature downshift cells were stained with propidium iodide to measure the DNA content by flow-cytometric analysis (FACS). Cells were detached with trypsin-EDTA, washed twice with ice-cold PBS and counted using the coulter counter as described above. Subsequently, 1×10^6 cells were resuspended in 500 μL cold PBS. To this suspension, 12 μL RNase A (10 mg/mL), 60 μL propidium iodide (50 μg/mL in 50 m*M* sodium citrate, pH 7.6) and 115 μL lysis buffer (0.75 % Nonidet P40; 5 m*M* EDTA; 5 m*M* EGTA; 0.1× YOPRO buffer) were added (5× YOPRO buffer: 100 m*M* sodium citrate, pH 4.0; 134 m*M* NaCl).

Cells were lysed on ice in the dark for 30 min prior to FACS analysis. Propidium iodide-mediated fluorescence is proportional to the cellular DNA content based on the stoichiometric affinity of this DNA intercalating dye. All FACS analyses were performed on a FACStar^{Plus} using the Cell Quest^{TM} software (Becton-Dickinson, San Jose, CA).

Two-Dimensional Gel Electrophoresis

At indicated time points, cells were harvested by scraping into cold-cell dissolvation solution (SIGMA) and washed three times with ice-cold PBS. After counting, cells were resuspended in 50 μL Z-buffer (10 m*M* Tris HCl (pH 6.8), 2% β-mercaptoethanol (BME), and 1% Nonidet P40). The cell suspension was sonified at 4°C for 120 s at 85% output (Branson Sonifier 450). The resulting lysates were heated to 95°C for 5 min to denature all proteins, and then immediately stored at 80°C until the first-dimension gel was loaded. Prior to loading, samples were mixed with an equal volume of solution C (9*M* urea, 2% BME, 2% NP-40, 0.8% BioLyte (pH3-10) carrier ampholytes (BioRad, Hercules, California), and 0.002% bromophenol blue). Two samples were mixed with a quantity of 2-D gel standards (BioRad) to determine approximate molecular weights and pIs of protein spots.

Equilibration, size separation, detection, and gel analysis were all performed as described previously (Lee et al., 1996). The gels were all silver-stained for protein detection. Isoelectric focusing was slightly different from the previously described protocol, and was performed as follows (old values in brackets): pH 3–10-nonlinear strips were used instead of the linear strips; the rehydration solution contained 0.333% [0.385%] BioLyte [pH 3–10], and 0.167% BioLyte [pH 5–7] (instead of 0.166% ServaLyte [pH 3–10]); the heat-transfer fluid between the DryStrip tray and the alignment card was Plusone DryStrip cover fluid (Pharmacia Biotech, Brussels, Belgium) instead of paraffin oil; the gels were run for a total of 71750 [72750] V h, with the voltage ramped linearly from 500 to 3500 V during the first 5 h [3 h] and maintained for 15.5 h [17.5 h] at 3500 V.

Western Blot Analysis

For analysis of tyrosine phosphorylated proteins, cells were solubilized in NP-40 extraction buffer (50 m*M* Hepes, pH 7.4, 150 m*M* NaCl, 25 m*M* β-glycero-phosphate, 25 m*M* NaF, 5 m*M* EGTA, 1 m*M* EDTA, 1% NP-40, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 m*M* PMSF, 1 m*M* sodium orthovanadate) for 10 min on ice. The lysates were clarified by centrifugation at 16,000*g* for 15 min. Protein concentrations were determined by Bradford assay. Seventy μg of total protein were subjected to SDS-PAGE (7.5% gel) and then blotted onto polyvinylidene difluoride membranes. After blocking with 20% horse serum (Gibco BRL) in TTBS (50 m*M* Tris, pH 7.5, 150 m*M* NaCl, 0.05% Tween-20), filters were probed with a phosphotyrosine-specific monoclonal antibody (Druker et al., 1989; Upstate Biotechnology, clone 4G10). Proteins were visualized with peroxidase-coupled secondary antibody using the ECL detection system (Amersham).

RESULTS

Growth Behavior and Morphology of XM111-10 Cells at 30°C

In CHO-K1 derived XM111-10 cells, secreted alkaline phosphatase (SEAP) is expressed in a tetracycline-dependent manner under the control of the $P_{hCMV*-1}$ promoter (Mazur et al., 1998; Mazur et al., submitted). In the present study, cells were grown without tetracycline in the culture medium to obtain SEAP secretion at the maximum level. To characterize the growth behavior of XM111-10 at different temperatures, we compared a low-temperature cultivation to the standard 37°C cultivation. Cultures were initiated by seeding 2×10^5 cells into T25 flasks, and cells were counted every 24 h for a period of 5 d. In the low-temperature cultivation, cells were shifted 48 h post-seeding from 37°C to 30°C. After the decrease in temperature growth of XM111-10 cells was almost completely arrested (Fig. 1), leading to a four times higher cell count in the 37°C culture compared to the 30°C culture at day 5 (72 h post-shift). CHO-K1 wild-type cells showed a similar growth behavior when exposed to low temperature (data not shown). After 5 d at 30°C, XM111-10 cells show changed morphology; cells were somewhat elongated compared to the round cells grown at 37°C (Fig. 2).

Cell-Cycle Distribution of XM111-10 Cells at 30°C

To further investigate the growth suppression of XM111-10 cells at 30°C, we quantified the cell-cycle distribution during a temperature shifted process by flow cytometric analysis. Cells were grown to early exponential phase and then shifted to 30°C. Cells of independent cultivations were harvested at indicated time points to survey the time course of growth inhibition at low temperature.

Prior to temperature downshift, 51% of the cells were in S phase, 36% were found in G1 phase, and 13% in G2/M (Fig. 3). When cells were harvested 48 h post-shift, 68% of the cells were in G1 vs. 23% in S and 10% in G2/M phase of the cell cycle. Exposure of XM111-10 cells to low temperature for 72 h resulted in accumulation of 79% of cells in G1 while 11% remained in S phase and 10% in G2/M (Fig. 3). These data clearly demonstrate that G1 phase is prolonged when XM111-10 cells are grown at 30°C. Accumulation of cells in G1 occurs within 2–3 d of cultivation, and cells were shown to be viable for at least 1 week post-shift, a time scale that makes a low-proliferation bioprocess feasible.

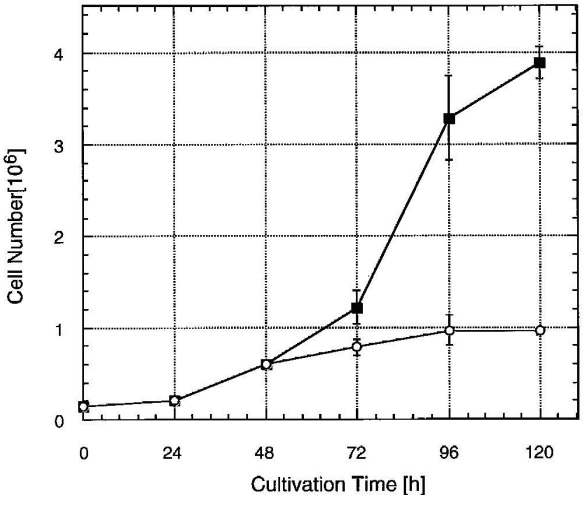

Figure 1. Comparison of growth behavior of XM111-10 cells grown at different temperatures. Cells were seeded at an initial cell density of 2×10^5 cells into T25 flasks and the cell number was measured daily for a period of 5 d. Low temperature cultivations were shifted from 37°C to 30°C 48 h post-seeding (○). Standard cultivations were grown for the entire period at 37°C (■).

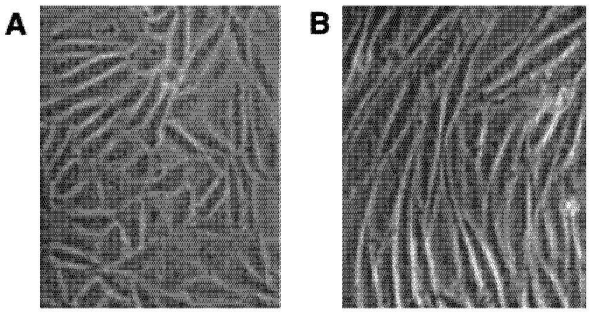

Figure 2. Morphology of XM111-10 cells at 37°C (A) and 5 d post-shift to 30°C (B).

Specific SEAP Productivity of XM111-10 Cells at 30°C

In the absence of tetracycline XM111-10 cells constitutively synthesize SEAP and secrete it into the culture medium (Mazur et al., submitted). To assess the influence of low temperature on $P_{hCMV*-1}$-directed expression and secretion of SEAP, we quantified the amount of SEAP in the culture medium for cultivations with and without temperature shift (see Fig. 1). Specific SEAP productivity values were obtained by normalizing the increase of product protein in the medium within a 24 h interval to 1×10^6 cells. When cells were grown at 37°C for the entire cultivation, specific SEAP productivity reached its peak values between 48 h and 72 h post-seeding (Fig. 4). This value was set to 100% and was determined to be 540 mU day^{-1} $(1 \times 10^6 \text{ cells})^{-1}$. On culture day 5, cells produced fivefold less SEAP. The specific productivity of cells grown at 30°C after 48 h increased up to 168 ± 3% compared to the 37°C productivity maximum, remaining at 154 ± 5% for another 24 h (Fig. 4). On culture-day 5, specific productivity at 30°C was eight times higher compared to the value obtained for the same day at 37°C.

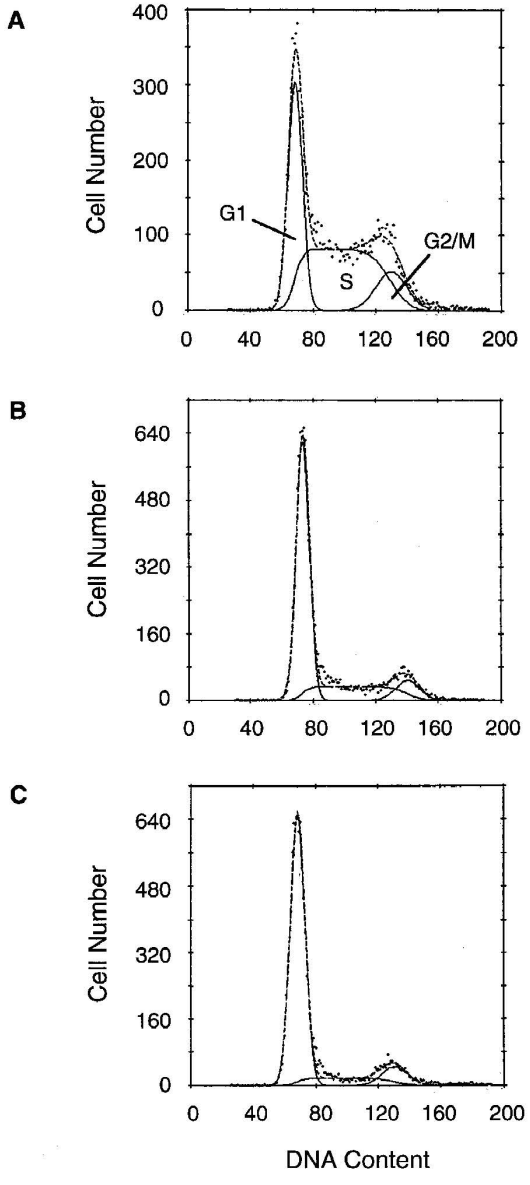

Figure 3. Cell-cycle distribution of XM111-10 cells in temperature-shift cultivations. Cells were grown at 37°C for 48 h and subsequently, temperature shifted to 30°C. Cells were harvested prior to shift (A), 48 h post-shift (B) and 72 h post-shift (C), stained with propidium iodide and analyzed for their DNA content by flow cytometry.

SEAP Production in a Temperature-Shifted-Batch Cultivation

The increase of specific SEAP productivity seen in XM111-10 cells grown at 30°C prompted us to determine the maximal product yield that could possibly be obtained at low temperature. We, therefore, shifted cells at higher densities to low temperature which resulted in a greater number of producing cells and terminated the cultivation only after some cells have started to enter death phase.

To quantify overall production 1×10^5 cells were seeded per T25 flask and grown at 37°C for 72 h before cultures were shifted to 30°C. SEAP accumulation in the medium was assayed and compared to the value obtained for the 37°C standard cultivation (Fig. 5). When cells were grown at 37°C, the maximum cell number was reached after 120 h. Cultures were terminated after 144 h when they entered death phase. In contrast, cultivation at 30°C prolonged the overall cultivation time up to 9 d. The maximum product amount for the 37°C process was set to 100% (154 mU/mL). In the low temperature cultivations SEAP accumulated up to 342 ± 4% after 9 culture days (Fig 5). Thus, approximately 3.5-fold higher final product titer is obtained with CHO XM111-10 cells in 30°C cultures.

Proteome Analysis of CHO Cells Grown at Different Temperatures

To survey the cold response of the CHO-cell proteome, we applied two-dimensional gel electrophoresis. CHO-K1 cells were grown at 37°C and shifted to 30°C after reaching exponential growth phase. Protein extracts from cells (5×10^6 cells per gel) harvested prior to shift and 48 h post-shift were compared. Proteins were visualized by silver-staining. In additional experiments, we separated 2-D standards (Biorad) alone and added to the samples. Superposition of these standard gels with the gels obtained for different temperatures provided the molecular weight and isoelectric point (pI) values marked in Figure 6. Computer-aided image analysis to compare 2-D electrophoresis patterns was performed using Melanie™ software. Care was taken to consider the relative overall darkness of certain gel regions to

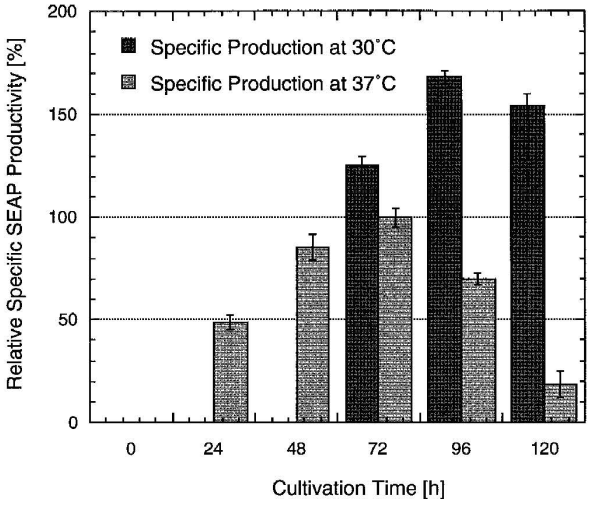

Figure 4. Relative-specific-SEAP productivity of XM111-10 cells at 30°C. The productivity data correspond to the growth behavior shown in Figure 1. SEAP concentration in the medium was measured every 24 h, and the daily increase was normalized to 1×10^6 cells to give specific productivity. The maximum specific productivity during standard cultivation at 37°C was set to 100% (540 mU d^{-1} (1×10^6 cells)$^{-1}$).

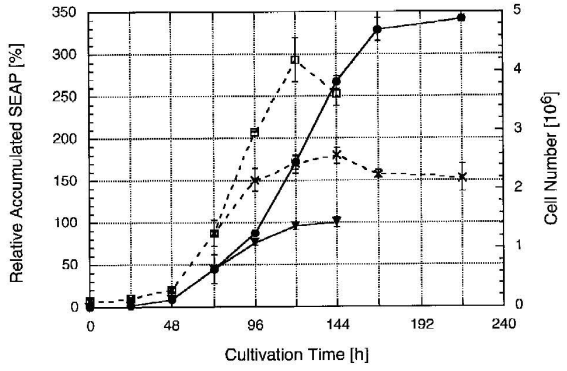

Figure 5. Cell proliferation and SEAP production in a temperature-shift-batch cultivation. XM111-10 cells were seeded at an initial cell density of 1×10^5 cells into T25 flasks, and the cell number was measured daily. Low-temperature cultivations were shifted from 37°C to 30°C 72 h post-seeding (✕). Standard cultivations were grown for the entire period at 37°C (□). Simultaneously, the SEAP concentration was measured in low temperature (●) and 37°C cultures (▼). The final SEAP yield of the 37°C cultivation was set to 100% (154 mU/mL).

eliminate effects caused by staining differences. Cold exposure clearly changed the overall protein expression patterns (Fig. 6). Spots that show significant changes in intensity after 48 h of cold exposure are highlighted in Fig. 6B. Six spots appeared darker on the 2-D image of extracts from the shifted cells. Three spots were detected with lower intensity on the same gel image. In one case, a spot clearly visible on the 37°C gel (Fig. 6B) was completely undetectable in extracts from cold-exposed cells. The changes in spot intensities seen in the 2-D images represent altered levels of a specific set of proteins when CHO cells are exposed to low temperature. In addition, we compared our gel images to a CHO-K1 reference protein map to match previously identified proteins (Lee et al., 1996). We were able to determine the location of HSP 90, HSC 70, HSP 60, β-Actin, α-Tubulin, and β-Tubulin (Fig. 6A). None of these proteins was subject to up- or downregulation due to cold exposure. However, our experiments demonstrate that low-temperature exposure of CHO cells changes the expression level of other specific proteins.

Influence of Low Temperature on Tyrosine-Phosphorylated Proteins in CHO Cells

Many signals are transmitted from the cell surface to the nucleus by kinase cascades, which trigger gene expression as a response to external stimuli. To obtain further insight into molecular pathways governing cold response of mammalian cells we surveyed the tyrosine-phosphorylation pattern in CHO cells when exposed to low temperature. CHO-K1 cells were grown at 37°C to exponential growth phase and subsequently shifted to 30°C.

Because both cell–cell contact and low temperature lead to growth suppression and may result in similar phosphorylation events, we included control extracts from cells which were kept at 37°C for an additional 24 h. We were thereby able to distinguish between the effect of cell–cell contact and low temperature on tyrosine-phosphorylated proteins. Proteins of CHO-K1 cells at 30°C were extracted at 24 h, 48 h, and 72 h post-shift. Extracts were examined for the presence of tyrosine-phosphorylated proteins by Western blot analysis using a phosphotyrosine-specific monoclonal antibody (Druker et al., 1989). Prior to the shift, five strong phosphotyrosine-containing bands were visible (Fig. 7, lane 1). In control cells at 37°C 24 h post-shift, increased tyrosine phosphorylation of a protein of approximately 160 kDa was observed (Fig. 7, lane 2). An increase in intensity of this band was also evident when extracts from cells grown at 30°C for 48 and 72 h were blotted (Fig. 7, lanes 4 & 5). However, two tyrosine-phosphorylated bands of approximately 120 and 140 kDa present also in control cells at 37°C demonstrated a more dramatic increase when cells were shifted to 30°C (Fig. 7, lane 2 vs. lane 5). A strong band corresponding to an approximately 40 kDa ty-

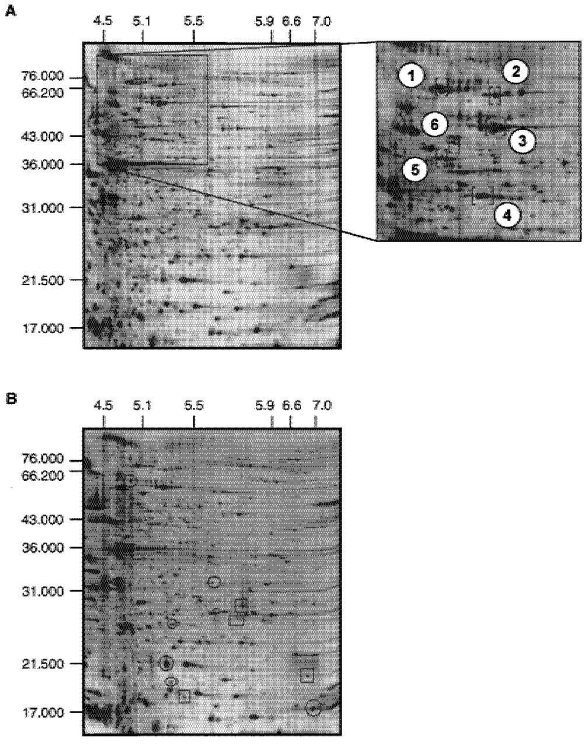

Figure 6. Alterations in the proteome of CHO cells following shift to low temperature. Whole-cell extracts of 5×10^6 CHO cells grown at 37°C (A) and 48 h post-temperature shift to 30°C (B) were separated by 2-D gel electrophoresis, and proteins were visualized by silver-staining. Spots that show significant changes in intensity after 48 h of cold exposure are highlighted in Figure 6B. Spots that appeared darker on the 2-D image are highlighted by ovals, while rectangles mark spots that were detected with lower intensity. Mobilities of coelectrophoresed-size markers are indicated on the left. pI marker values are indicated on the horizontal axis. Numbers in the enlarged gel section (A) refer to proteins identified by comparison to a CHO-K1 reference map (Lee et al., 1996): HSP 90 **(1),** HSC 70 **(2),** HSP 60 **(3),** β- and γ- Actin **(4)** β-Tubulin **(5)** and α-Tubulin **(6).**

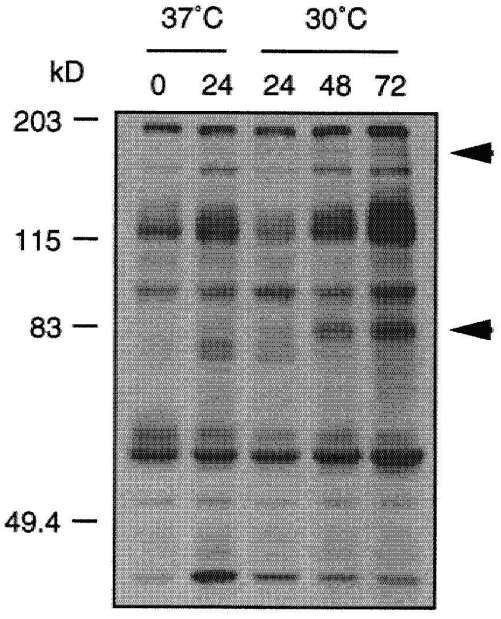

Figure 7. Influence of low temperature on tyrosine-phosphorylated proteins in CHO cells. Cells were harvested at indicated times (h) after temperature shift from 37°C to 30°C. The time point 37°C 0 h (lane 1) corresponds to the initial point of the 30°C culture. 70 μg of samples were separated by 7.5% SDS-PAGE. Membranes were probed with a phosphotyrosine-specific monoclonal antibody. Mobilities of coelectrophoresed size markers are indicated on the left. Arrows indicate low temperature specific bands.

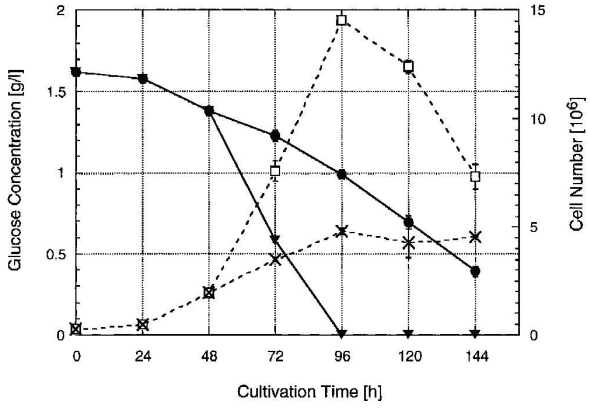

Figure 8. Cell proliferation and glucose consumption in a temperature-shift-batch cultivation. CHO cells were seeded at an initial cell density of 2.5×10^5 cells into T75 flasks and the cell number was measured daily. Low temperature cultivations were shifted from 37°C to 30°C 48 h post-seeding (✕). Standard cultivations were grown for the entire period at 37°C (□). Simultaneously, the glucose concentration was measured in low temperature (●) and 37°C cultures (▼).

rosine-phosphorylated protein occurred at 37°C 24 h post-shift, but was only weakly induced at 30°C. Interestingly, an approximately 80 kDa protein-demonstrated phosphorylation on tyrosine at low temperature which steadily increased during the 72 h time course. Similarly, but to a lower extent, a 180 kDa protein showed elevated levels of phosphotyrosine after 72 h at 30°C (Fig. 7, lanes 3–5). On the other hand, no tyrosine phosphorylation of these two proteins was observed when cells were harvested at 37°C 24 h post-shift (lane 2), indicating that these phosphorylation events are specific to cells grown at low temperature.

Glucose Consumption of CHO Cells in Low-Temperature Cultivations

To exclude medium depletion as a possible factor in the appearance of the two tyrosine-phosphorylation specific bands (Fig. 7), we quantified the glucose concentration in the culture medium for cultivations with and without temperature shift. Cultures were initiated by seeding 2.5×10^5 cells into T75 flasks and cells were counted every 24 h for a period of 6 d. In the low-temperature cultivation, cells were shifted 48 h post-seeding from 37°C to 30°C. The concentration of glucose in the culture medium was measured simultaneously (Fig. 8). The glucose concentration in culture medium 72 h post-shift to 30°C was 0.69 g/L compared to 0.58 g/L when cells grew for another 24 h at 37°C. These data indicate that medium depletion does not play a role in the induction of two tyrosine-phosphorylated proteins at low temperature seen in our immunoblot experiments (Fig. 7).

DISCUSSION

CHO cells are widely used for large-scale production of human recombinant proteins for therapeutic use. Production of heterologous proteins at low temperature is discussed as an approach to improve mammalian batch-culture performance. In this study, the CHO K-1 derived-cell line XM111-10, engineered to produce SEAP under the control of the cytomegalovirus derived P_{hCMV^*-1} promoter, was used as a model system to investigate the influence of low-cultivation temperature on heterologous protein production. We characterized the growth behavior of XM111-10 cells at low temperature by shifting cells to 30°C at different cultivation stages. In all experiments, the growth rate decreased significantly after temperature shift to 30°C, with growth suppression occurring more rapidly when cells were shifted at low densities. These results emphasize that temperature can be used as a means to control proliferation of mammalian cells.

FACS analysis showed that approximately 80% of cells accumulated in the G1 phase of the cell cycle 72 h after shift to 30°C. The G1/S transition is the most important restriction point in the mammalian cell cycle and its control is essential in many cellular processes such as embryonic development and cancer. It is tempting to speculate that cells respond actively to the decrease in temperature by halting cell-cycle progression at the G1/S boundary before DNA replication occurs. However, we found 10% of the cells to

reside in the G2/M phase of the cell cycle 72 h post-shift, indicating that cold-induced growth arrest might not only occur at a singular check point.

We next compared the specific SEAP productivity of XM111-10 cells following shift to 30°C to the maximum productivity value of cells grown at 37°C for the entire cultivation period. Our data show that in addition to increasing specific productivity for our system, low temperature prolongs cultivation time in batch cultures, and thereby further increases the overall amount of SEAP accumulated. According to previous studies, low temperature does not generally have a positive effect on recombinant-protein production and can, in some cases, result in decreased productivity (Bloemkolk et al., 1992; Giard et al., 1982; Reuveny et al., 1986; Sureshkumar and Mutharasan, 1991; Weidemann et al., 1994). Recently, enhanced C-terminal α-amidating enzyme production was observed in CHO cells grown at 30°C (Furukawa and Kazuhiro, 1998). These data are consistent with our findings and support the hypothesis that diverging results regarding productivity at low temperatures might be explained by different host-cell lines and/or promoters used.

Recently, we showed elevated SEAP production in $p27^{KIP1}$ overexpressing CHO cells (Fussenegger et al., 1998, Mazur et al., 1998; Mazur et al., submitted). Similar to cold exposed XM111-10 cells, these cells exhibit their increase in productivity while residing in the G1 phase of the cell cycle. Expression of the *seap* gene was driven by the $P_{hCMV*-1}$ promoter in both cases. $P_{hCMV*-1}$ consists of a minimal promoter sequence derived from the human cytomegalovirus (CMV) promoter IE combined with a multiple-tet operator site encoded in Transposon 10 of *E. coli*. In the absence of tetracycline the transactivator (tTA), which is constitutively expressed in XM111-10 cells, binds the tet-operator sequences and thereby, activates the promoter. Constitutive tTA expression in XM111-10 cells is driven by the full-length cytomegalovirus (CMV) promoter. Interestingly, it was recently reported by several groups that human cytomegalovirus infection inhibits cell-cycle progression, including the transition from G1 to S (Bresnahan et al., 1996; Dittmer and Mocarski 1997; Jault et al., 1995; Lu et al., 1996). Presumably, host-cell proliferation is blocked prior to S phase to provide a favorable environment for expression of viral genes. This could explain high activity of the CMV-derived $P_{hCMV*-1}$/tTA expression system in cells that reside in the G1 phase of the cell cycle. However, it remains to be investigated whether elevated transcription is responsible for the increase of SEAP secretion seen in cold exposed or p27 overexpressing CHO cells.

A temperature-shift process divides cultivation into a growth phase at 37°C and a low-proliferation phase at 30°C. Sometimes it is desirable to induce gene expression at a certain stage of the cultivation, for instance, when a gene product is expressed that inhibits growth of the host cell. In this case, separation of the process into a proliferation and a production phase would be the ideal solution. We expect our model system to be especially suitable for establishing such a biphasic bioprocess because product synthesis in XM111-10 cells can be completely suppressed by addition of tetracycline to the medium (Mazur et al., submitted). After an initial proliferation-phase withdrawal of tetracycline from the medium accompanied by a temperature downshift will suppress growth and initiate SEAP production. Similar processes can be established with cells engineered to coexpress growth inhibitors such as p27 together with the product gene in a tetracycline regulatable manner. However, a major problem in controlling proliferation in a production process by genetic engineering is the occurrence of mutants which escape growth suppression (Mazur et al., submitted). Mutation or loss of the genes necessary for proliferation control will provide a substantial growth advantage to mutant cells which can lead to a high number of non-producing cells and subsequent loss in product yield. On the contrary, no fast growing mutants were observed when proliferation of XM111-10 cells was controlled by low temperature.

The molecular response of mammalian cells to low temperatures between 27°C and 32°C is not well-understood. The discovery of the glycine-rich RNA-binding protein CIRP provided first evidence for induction of specific proteins in cold-exposed murine fibroblasts (Nishiyama et al., 1997). However, it still remains to be elucidated whether mammalian cells generally respond in an active manner by induction of cold-specific proteins. To test whether distinct proteins are subject to up- and downregulation due to a drop of cultivation temperature, the CHO proteome was analyzed by 2-D polyacrylamide electrophoresis of whole cell extracts from CHO cells grown at 37°C and from cells that were shifted for 48 h to 30°C. The 30°C 2-D gel images showed 10 spots with significantly higher or lower intensity compared to the 37°C standard. These experiments demonstrate that some proteins do, indeed, exhibit altered expression levels in mammalian cells after temperature downshift.

Stress signals must be transduced into biochemical changes to alter gene expression patterns. Such transduction is commonly mediated, in part, by a number of intracellular kinase and phosphatase enzymes. One group among them are the stress-activated protein kinases (SAPKs) or alternatively the c-jun *N*-terminal kinases (JNKs) and p38 MAP kinase (the mammalian homologue of yeast HOG1) (Paul et al., 1997). These kinases are activated in response to a number of cellular stresses including inflammatory cytokines, bacterial endotoxins, ischaemia/cellular ATP depletion, and heat and chemical shock. In stress-kinase pathways the signal is transduced by altering the phosphorylation state of tyrosine residues of target proteins. We used a phosphotyrosine-specific monoclonal antibody (Druker et al., 1989) to investigate the role of tyrosine phosphorylation in the cold response of CHO cells. Western blot analysis showed several tyrosine-phosphorylated proteins to be induced between 48 h and 72 h post-shift to 30°C. To distinguish between phosphorylation caused by contact inhibition in confluent cultures and temperature-specific effects we included extracts from cells grown another 24 h at 37°C (37°C 24 h).

Comparison of immunoblotted extracts revealed low-temperature-specific tyrosine phosphorylation of two proteins of approximately 180 kD and 80 kD, respectively. Additional experiments showed that 48 h and 72 h after the shift to low-temperature glucose concentration is higher than in the medium of the control cells (37°C 24 h) indicating that it is unlikely that medium depletion plays a roll in the induction of the two phosphorylated proteins.

To our knowledge, this report provides the first evidence for low-temperature-specific changes at a post-translational level in mammalian cells. The two proteins demonstrating cold-induced tyrosine phosphorylation may represent key regulators in mammalian cold response and remain to be identified. An important cellular event after decrease in cultivation temperature seems to be suppression of cell growth, preferably by halting the cell cycle in G1. It will be of great interest to identify the molecules which trigger this active-cellular response.

In summary, low-temperature cultivation is a means to control cell proliferation and can serve as a first step to establish two-stage mammalian bioprocesses. The decrease in temperature prolongs culture viability which is thought to be a result of delayed onset of apoptosis (Moore et al., 1997). Furthermore, temperature shift can lead to an increase in specific productivity and overall product yield. Determining molecular aspects of this stress response may, therefore, provide important information useful to further improve animal cell-culture performance.

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Hypothermia Causes a Reversible, p53-Mediated Cell Cycle Arrest in Cultured Fibroblasts

Zdenka Matijasevic,[1] John E. Snyder, and David B. Ludlum

Department of Pharmacology and Molecular Toxicology, University of Massachusetts Medical School, Worcester, MA 01655-0126

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Normal human fibroblasts grown in cell culture undergo a reversible growth arrest when incubated at 28°C. During incubation at 28°C, levels of p53 and p21 rise in these cells and cell cycle analysis shows that they have undergone a cell cycle arrest. To examine the importance of p53 in mediating this arrest, mouse embryo fibroblasts that are either wild-type or that are defective in p53 were also subjected to hypothermia. Only those cells with wild-type p53 undergo a cell cycle arrest, indicating that p53 has a role in mediating this response. Because many tumor cells have defective p53, this suggests that hypothermia may increase the selective toxicity of chemotherapeutic agents for tumor cells.

Key words: Hypothermia; Cell cycle arrest; p53 protein; Fibroblasts

Both hyper- and hypothermia have had wide applications in a variety of medical procedures and therapeutic regimens. Hyperthermia sensitizes tumor cells to ionizing radiation and to radiation combined with chemotherapy [reviewed in (1,2)]. The protective effect of mild hypothermia against neuronal damage following ischemia is well established (3,4) and has been attributed to hypothermia-modulated expression of specific immediate-early genes induced by ischemia (5,6).

The response of mammalian cells to elevated temperature as a single modality has been studied extensively during the past decade at the cellular and molecular levels. Progress has been made in understanding the role of heat shock proteins (HSPs) in transient thermotolerance, in permanent heat resistance [for review see (7)], and in protection against stress-induced apoptosis (8,9). In contrast to elevated temperatures, the present knowledge of molecular and biochemical consequences of downshifts in temperature comes primarily from studies of the cold shock response in *Escherichia coli* (10–12). Although the effect of low temperatures on growth and cell cycle progression was noted in mammalian cells as early as 1965 (13), the first known mammalian cold-inducible protein was identified only recently (14).

As reported here, we have found that hypothermia induces a cell cycle arrest and increases levels of p53 protein in normal human fibroblasts. Studies with mouse embryo fibroblasts have shown that in contrast to p53 wild-type cells, cells that are deficient in p53 fail to arrest at low temperature, indicating that hypothermia-induced cell cycle arrest of mammalian cells requires functional p53 protein.

MATERIALS AND METHODS

Cell Lines

The normal human diploid fibroblast cell line, AG01522, was obtained from the Aging Cell Repository, Coriell Institute for Medical Research (Camden, NJ) at a population doubling level (PDL) of 15 (PDL: number of cumulative population doublings from primary culture). Human fibroblasts were cultured as monolayers in minimum essential medium (MEM) supplemented with nonessential amino acids (ICN Pharmaceuticals, Costa Mesa, CA). Mouse embryonic fibroblast cells, wild-type for p53 and p53 null mutants, both at passage 2, were obtained from Stephen N. Jones, Department of Cell Biology, University of Massachusetts Medical Center (15,16). These cells were grown as monolayers in Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Gaithersburg, MD). Penicillin and streptomycin at concentrations of 100 IU/ml each and 15% heat-inactivated fetal calf serum were added to all media.

Culture Conditions and Viability Determinations

Cells were plated at a density of 2×10^4 cells/cm^2, and temperature experiments were performed with parallel sets of cells incubated at either 37°C or 28°C in humidified atmospheres with 5% or 4.6% CO_2, respectively. Cells were harvested by trypsinization for viability determinations by the trypan blue exclusion (TBE) assay. In order to include cells that may have been detached from the monolayer, the medium above the monolayer was collected and centrifuged prior to tryp-

[1]Address correspondence to Zdenka Matijasevic, Department of Pharmacology and Molecular Toxicology, University of Massachusetts Medical School, Worcester, MA 01655-0126. Tel: (508) 856-2459; Fax: (508) 856-5080.
[2]Abbreviations used: BrdU, 5-bromo-2′-deoxyuridine; FACS, fluorescence activated cell sorting.

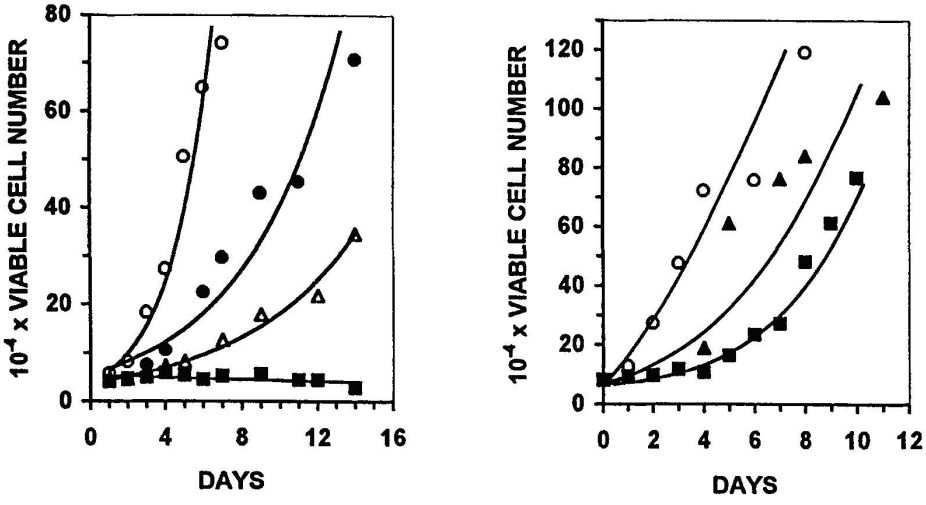

Figure 1. Effect of hypothermia on growth of normal human AG01522 fibroblasts. Left panel: cells were incubated continuously at 37°C (○), 33°C (●), 31°C (△), or 28°C (■). Right panel: cells were either incubated continuously at 37°C (○), or grown at 28°C for 2 (▲) or 4 (■) days and then transferred to 37°C. The viable cell number was determined by the trypan blue exclusion assay.

sinization, and the pellet was combined with cells detached by trypsin.

Cell Cycle Analysis

Cell cycle distribution was determined as DNA content per cell by propidium iodide staining. Approximately 1×10^6 cells were fixed with ethanol and kept at 4°C for at least 24 h before analysis. Prior to staining, low molecular weight DNA was extracted by the procedure described by Hotz et al. (17). This extraction allows identification of cell populations with fractional, sub-G_1 DNA content indicative of DNA fragmentation and cell death. Ethanol-fixed cells were pelleted and incubated for 30 min in pH 7.8 buffer consisting of 50 mM Na_2HPO_4 (9 parts) and 25 mM citric acid (1 part), containing 0.1% Triton X-100. Cells were then stained for 30 min at room temperature in 10 mM piperazine-*N,N'*-bis(2-ethanosulfonic acid) (PIPES) buffer, pH 6.8, containing 0.1 N NaCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 20 μg/ml propidium iodide, and 50 μg/ml RNase. Cell cycle analysis was performed using a Becton-Dickinson FACScan flow cytometer (Mountain View, CA). At least 15,000 events were collected per sample; cell doublets and aggregates were electronically eliminated from analysis. The percentage of cells in each phase of the cell cycle was determined using Modfit software (Verity Software House, Topsham, ME).

Replicative DNA synthesis was determined as 5-bromo-2′-deoxyuridine ($BrdU^2$) incorporation following a slightly modified procedure described by Jones et al. (15). Cells were labeled in 13 μM BrdU for 2 h, har-

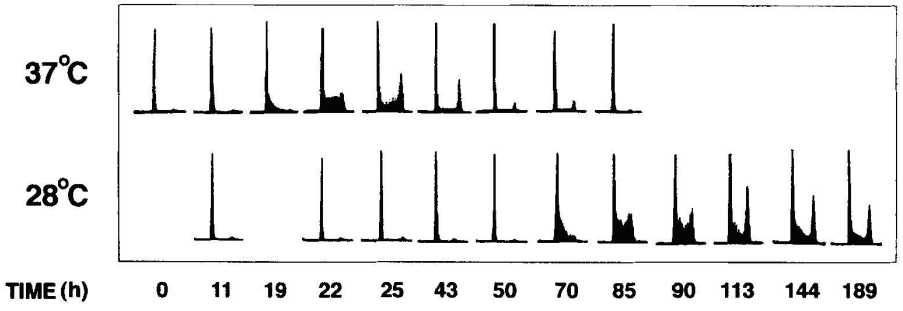

Figure 2. Effect of hypothermia on cell cycle progression in normal human AG01522 fibroblasts. Cells were plated at a density of 2×10^4 cells/cm^2 and incubated at either 37°C or 28°C; after staining with propidium iodide the distribution of DNA content was analyzed by FACS analysis. Top row: DNA profiles at 37°C; bottom row: DNA profiles at 28°C.

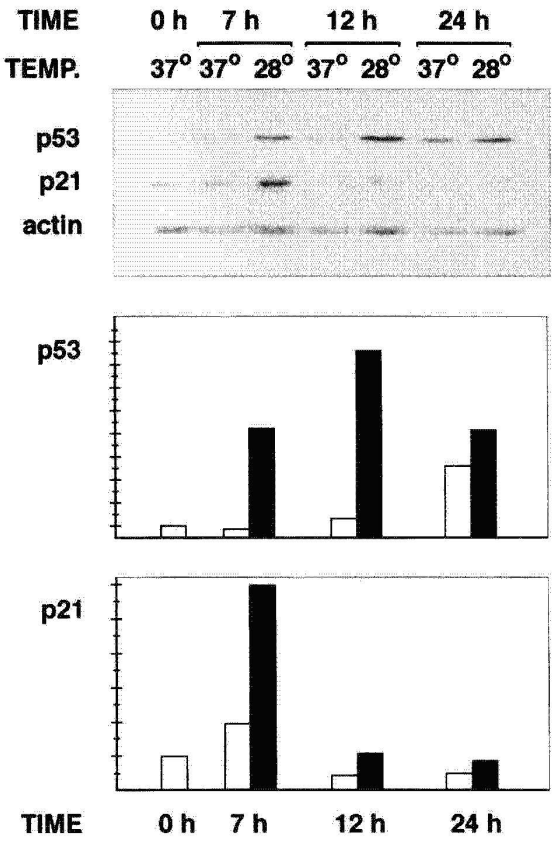

Figure 3. Levels of p53 and p21 in human fibroblasts grown at 37°C or at 28°C. Cells were plated at a density of 2×10^4 cells/cm^2 and incubated at either 37°C or 28°C. At the indicated times, cells were lysed and protein (50 µg) was separated by PAGE on 10% SDS gels. Western blot analyses (upper panel) were performed as described in the text. Levels of p53 (middle panel) and p21 (lower panel) were determined densitometrically. Plotted values are densities relative to β-actin. Open bars: 37°C; solid bars: 28°C.

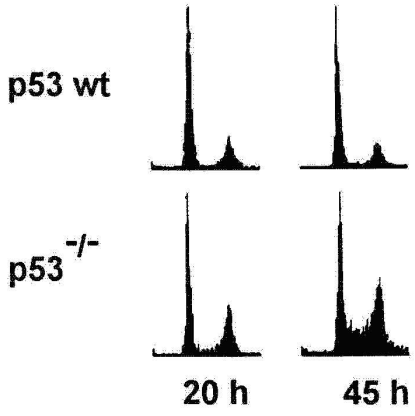

Figure 4. Effect of hypothermia on cell cycle progression in wild-type and p53-deficient mouse embryo cells. Cells were plated at a density of 2×10^4 cells/cm^2, incubated continuously at 28°C, and stained with propidium iodide for analysis of DNA content. Top row: wild type cells; bottom row: p53-deficient cells.

vested by trypsinization, fixed with 70% ethanol, and stored at −20°C until analysis. Samples were treated with 0.1 N HCl containing 0.5% Triton X-100 for 30 min at room temperature, boiled for 2 min, and rapidly cooled to denature DNA. Cells were then washed twice with 0.1 M $Na_2B_4O_7$ solution (pH 8.5), incubated with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibodies for 30 min (1:4 dilution; Caltag Laboratories, Burlingame, CA), and counterstained with propidium iodide as described.

Western Blot Analysis

For Western blot analysis, cells were harvested by trypsinization at either 37°C (for cells grown at 37°C) or at 28°C (for cells grown at 28°C), rinsed with PBS, and stored at −20°C until analysis. Samples were lysed on ice for 30 min in 50 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (Boehringer Mannheim, Indianapolis, IN), 100 KIU/ml aprotinin (Calbiochem, San Diego, CA), and 10 µg/ml leupeptin (Boehringer Mannheim). Cell lysates were centrifuged for 10 min at $15,600 \times g$ and the protein concentration in each supernatant was determined by the BioRad protein assay. Samples were boiled for 5 min in a loading buffer of 50 mM Tris-HCl, pH 6.8, containing 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.1% bromphenol blue, and 28 mM β-mercaptoethanol. Proteins (50 µg/lane) were resolved by electrophoresis in 10% polyacrylamide-SDS gels and transferred to a polyvinylidene fluoride membrane (BioRad, Hercules, CA) with a transfer buffer of 25 mM Tris-HCl containing 192 mM glycine and 10% v/v methanol. Biotinylated protein markers (New England Biolabs, Beverly, MA) were used as molecular weight standards. After transfer, the gel was stained with Coomassie blue as a control for loading and transfer. The membrane was probed with the p53-specific monoclonal antibody, Pab1800 (Oncogene Research Product-Calbiochem, Cambridge, MA), and then reprobed with the p21-specific antibody WAF1 (Ab-1) (Oncogene) and with the monoclonal anti-β-actin N350 antibody (Amersham Life Science, Arlington Heights, IL). Immunoreactive proteins were detected with alkaline phosphatase-conjugated anti-mouse immunoglobulin (IgG) and a chemiluminescent reagent (CDP-star) (New England BioLabs). After autoradiography, signals were quantitated by densitometric scanning with a Molecular Dynamics Personal Densitometer. Analysis was performed using the ImageQuant (version 1.1) program. Levels of p53 and p21 proteins were determined relative to the levels of β-actin.

RESULTS AND DISCUSSION

The effects of mild hypothermia on the growth of normal human fibroblast AG01522 cells are shown in

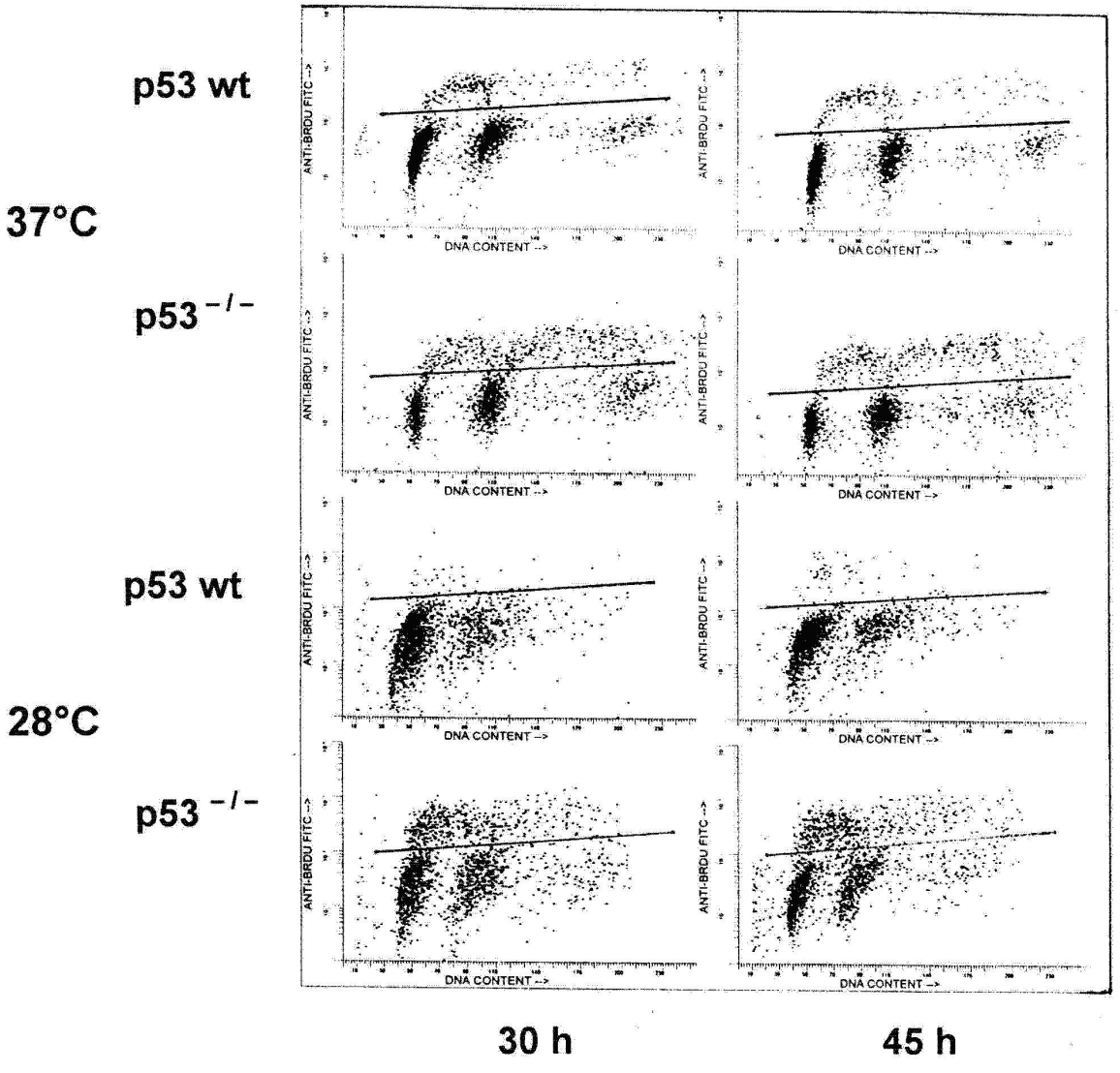

Figure 5. Effect of p53 status on cell cycle progression in mouse embryo fibroblasts incubated at 37°C or 28°C. Cells were plated at a density of 2×10^4 cells/cm^2 and incubated at either 37°C or 28°C. At the indicated times, the cells were labeled with BrdU for 2 h, fixed, stained with anti-BrdU-FITC and propidium iodide, and analyzed by flow cytometry. First row from the top: wild-type cells at 37°C; second row: p53-deficient cells at 37°C; third row: wild-type cells at 28°C; and bottom row: p53-deficient cells at 28°C.

Figure 1. The left-hand panel shows the growth of cells incubated at either 37°C, 33°C, 31°C, or 28°C. At 33°C and 31°C, the cell number increases more slowly than at 37°C, but at 28°C the cell number remains constant; there is no indication of cell death at the lower temperatures either by trypan blue exclusion criteria or from cell cycle analysis data. This suggests that the constant viable cell number in the population of AG01522 cells incubated at 28°C results from cell cycle arrest rather than from a balance between cell proliferation and cell death. The data in the right-hand panel of Figure 1 indicate that the growth arrest at 28°C is reversible; cells resume growth and proliferate with a normal growth rate after 2 or even 4 days at 28°C when the temperature is shifted back to 37°C.

We examined the effect of hypothermia on cell cycle progression by FACS analysis as shown in Figure 2. Fibroblasts were plated at a density of 2×10^4 cells/cm^2 and incubated continuously at either 37°C or 28°C; subsequently, cells were harvested and cell cycle analysis was performed as described. As seen from this figure, cell cycle progression into the S phase is delayed for at least 2 days in populations of AG01522 cells grown at 28°C. After 4 days, cells at 28°C start very slowly to

accumulate in the G_2/M phase and the fraction of cells in G_2/M increases on the expense of cells in G_1 from 12% on day 4 to 30% on day 8. A lack of increase in the percentage of cells in G_1 for the entire duration of the experiment suggests that cells do not progress into the next cell cycle. This is in agreement with the data on cell growth as determined by the TBE assay.

Cell cycle arrest is a cellular response common to many physiological stress conditions, but the pathways leading to arrest may be stress specific. G_1 arrest induced by DNA damaging agents is most often a manifestation of the p53-dependent transactivation of the cyclin-dependent kinase inhibitor, p21 (18–20). To examine the effects of mild hypothermia on cellular p53 and p21 protein levels in fibroblasts, Western blot analyses were performed with whole cell extracts from AG01522 cells incubated at either 37°C or 28°C. As shown in Figure 3, both p53 and p21 accumulated in cells incubated at 28°C. However, the time course for the accumulation of these two proteins was different. The highest level of p21, a threefold increase above the level at 37°C, was found in cells after 7 h of incubation at 28°C. The p53 levels peaked after 12 h and reached an eightfold increase above the level at 37°C. The levels of p21 protein had decreased before the cells were released from the G_1 block.

As suggested by other studies, the p53–p21 pathway may also be involved in cellular responses to elevated temperatures (21–23), and to cold shock (4°C for 60 min) (24). In contrast, hypoxia increases cellular p53 levels, but accumulation of cells in the G_1 phase by hypoxia is not p53 dependent (25). To test whether p53 is indeed required for the cell cycle arrest we observe at low temperature, cell cycle analyses were performed on wild-type and p53-deficient mouse embryo fibroblasts incubated at 28°C. The profiles of DNA content shown in Figure 4 indicate that p53-deficient cells progress through the cell cycle under hypothermic conditions whereas cells that are wild-type for p53 stay arrested for at least 2 days.

The requirement for p53 in cell cycle arrest by hypothermia was confirmed by measuring BrdU incorporation during the incubation of wild-type and p53-deficient mouse embryo fibroblasts at 28°C and 37°C. The results shown in Figure 5 demonstrate the suppression of replicative DNA synthesis in wild-type cells at 28°C for at least 45 h after plating. Even 65 h after plating (data not shown), fewer than 4% cells of the wild-type population incorporate BrdU at 28°C. In contrast, nearly 30% of the p53-deficient cell population undergo DNA synthesis at 28°C. One fraction of BrdU-incorporating p53-deficient cells has a higher than diploid DNA content, and probably reflects the DNA rereplication previously demonstrated for cells lacking p53 (26). A fraction of hyperdiploid cells is present in the populations of cells grown at both temperatures and is a growth characteristic of p53-deficient mouse embryo fibroblasts (27).

In conclusion, we have shown that mammalian cells undergo a reversible cell cycle arrest in response to mild hypothermia. Because p53 appears to be required for this arrest and many tumor cells lack functional p53, normal cells would presumably arrest while p53-deficient tumor cells would continue to cycle under hypothermic conditions. This hypothermia-induced cell cycle arrest could protect normal cells and might magnify the selective toxicity for tumor cells of antitumor agents that act on proliferating cells.

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online data

ATR (ataxia telangiectasia mutated- and Rad3-related kinase) is activated by mild hypothermia in mammalian cells and subsequently activates p53

Anne ROOBOL*, Jo ROOBOL*, Martin J. CARDEN*, Amandine BASTIDE†, Anne E. WILLIS†[1], Warwick B. DUNN‡, Royston GOODACRE‡ and C. Mark SMALES*[1]

*Centre for Molecular Processing and Protein Science Group, School of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, U.K., †MRC Toxicology Unit, Hodgkin Building, P.O. Box 138, University of Leicester, Lancaster Road, Leicester LE1 9HN, U.K., and ‡School of Chemistry and Manchester Centre for Integrative Systems Biology, Manchester Interdisciplinary Biocentre, University of Manchester, Manchester M1 7DN, U.K.

In vitro cultured mammalian cells respond to mild hypothermia (27–33 °C) by attenuating cellular processes and slowing and arresting the cell cycle. The slowing of the cell cycle at the upper range (31–33 °C) and its complete arrest at the lower range (27–28 °C) of mild hypothermia is effected by the activation of p53 and subsequent expression of p21. However, the mechanism by which cold is perceived in mammalian cells with the subsequent activation of p53 has remained undetermined. In the present paper, we report that the exposure of Chinese-hamster ovary-K1 cells to mildly hypothermic conditions activates the ATR (ataxia telangiectasia mutated- and Rad3-related kinase)–p53–p21 signalling pathway and is thus a key pathway involved in p53 activation upon mild hypothermia. In addition, we show that although $p38^{MAPK}$ (p38 mitogen-activated protein kinase) is also involved in activation of p53 upon mild hypothermia, this is probably the result of activation of $p38^{MAPK}$ by ATR. Furthermore, we show that cold-induced changes in cell membrane lipid composition are correlated with the activation of the ATR–p53–p21 pathway. Therefore we provide the first mechanistic detail of cell sensing and signalling upon mild hypothermia in mammalian cells leading to p53 and p21 activation, which is known to lead to cell cycle arrest.

Key words: ataxia telangiectasia mutated- and Rad3-related kinase (ATR), Chinese-hamster ovary cell (CHO cell), cold shock, hypothermia, lipidomics, metabolomics, p53.

INTRODUCTION

Under mildly hypothermic conditions (31–33 °C), mammalian cells proliferate slowly [1] and generally attenuate the processes of transcription and mRNA translation [2] (although protein folding may actually improve [3]), and the cell cycle proceeds at a much reduced rate [4]. However, below 30 °C, cells become arrested, predominantly in G_1 phase [5,6], normally the stage in the cell cycle when protein synthesis rates are optimal. Indeed, because of this, other strategies for inducing cell cycle arrest in late proliferative stage cultures of mammalian cells in an industrial sense have been investigated, including generation of cell lines with inducible expression of the general cyclin inhibitor p21 [7] and the addition of solvents, such as DMSO, to the growth medium, which also induces p21 expression [8]. However, exposure to mildly hypothermic conditions remains the most economic and most effective way of extending the productive life of cultured mammalian cells for large-scale recombinant protein production [9].

The slowing of the cell cycle at the upper range of mild hypothermia (31–33 °C) and its complete arrest at the lower range of mild hypothermia (27–28 °C) is regulated by the expression of p21 [10]. There are numerous examples (reviewed in [11]) of p21-induced cell cycle arrest protecting damaged or stressed cells from apoptosis, thus providing a time window within which the damage may be repaired or stress conditions removed. This is certainly the case for mildly cold-stressed cells, since they recover rapidly and fully on returning to 37 °C [12]. It is also well established that p21 induction in mildly hypothermic cells is subsequent to an increase in the stability and hence amounts of the tumour suppressor protein p53 [10,13] and to changes in the p53 isoform array [12], although the post-translational modification(s) generating these observed changes in p53 isoform pattern remain to be identified. Indeed, p53-deficient mammalian cells do not show cell cycle arrest at mildly hypothermic temperatures, confirming the key role of p53 in regulating this process upon cold shock [10,13]. However, the mechanism(s) by which p53 phosphorylation and amounts are unregulated upon mammalian cells being placed under hypothermic conditions, or how these conditions are sensed, are currently unknown.

In addition to cell cycle arrest and the general attenuation of transcription and translation, changes to the cell membrane composition are also observed when both prokaryotic [14] and eukaryotic [15] cells are exposed to hypothermic conditions. Essentially, cells respond to reduced temperature by increasing the polyunsaturated fatty acid content of membrane phospholipids, thereby maintaining the fluidity under hypothermic conditions, so-called homeoviscous adaptation [16]. At 37 °C, an increase in polyunsaturated fatty acid content of membrane phosphatidylcholines, induced by exposure to the Ca^{2+}-dependent phospholipase A_2 inhibitor BEL (bromoenol lactone), has been reported to arrest mammalian cells in G_1-phase by activation of the p53–p21 pathway [17]. This was subsequently shown to be mediated by phosphorylation of p53 at Ser^{15} by a member of

Abbreviations used: ATM, ataxia telangiectasia mutated; ATR, ATM- and Rad3-related kinase; BEL, bromoenol lactone; CHO, Chinese-hamster ovary; CIRP, cold-inducible RNA-binding protein; DMEM, Dulbecco's modified Eagle's medium; DNA-PK, DNA-dependent protein kinase; FBS, fetal bovine serum; mTOR, mammalian target of rapamycin; Mdm2, murine double minute 2; $p38^{MAPK}$, p38 mitogen-activated protein kinase; PC, principal component; PC-DFA, principal component-discriminant function analysis; PIKK, phosphoinositide 3-kinase-related kinase; qRT-PCR, quantitative real-time PCR; RNAi, RNA interference; siRNA, small interfering RNA; UTR, untranslated region.

[1] Correspondence may be addressed to either of these authors (email aew5@le.ac.uk or c.m.smales@kent.ac.uk).

the PIKK (phosphoinositide 3-kinase-related kinase) family, ATR (ataxia telangiectasia mutated- and Rad3-related kinase) [17], a signalling pathway more usually associated with cell cycle arrest in response to compromised DNA replication [18]. Since there was no evidence of DNA damage in these cells with altered membrane composition, it was concluded that an increase in the ratio of polyunsaturated to saturated fatty acids in phosphatidylcholines in cell membranes independently activates the ATR–p53–p21 pathway [17]. Furthermore, the expression of the cold-shock protein CIRP [cold-inducible RNA-binding protein, also known as hnRNP (heterogeneous nuclear ribonucleoprotein) A18] is induced at mildly hypothermic temperatures in mammalian cells [19] and binds to the 3′-UTRs (untranslated regions) of certain transcripts, increasing their translation [20]. CIRP protein binds to the 3′-UTR of *ATR* mRNA, and overexpression of CIRP results in increased ATR protein levels [20].

In view of these reports and our own previous observations that changes in p53 isoform pattern are observed upon mildly hypothermic conditions in mammalian cells as are the detection of lipid droplets at lower temperature [12], we set out to investigate (i) whether p53 activation upon mild hypothermia was at least in part mediated through the ATR kinase signalling pathway, and (ii) whether mild hypothermia resulted in changes in lipid composition consistent with those previously reported to activate ATR (an increase in the ratio of polyunsaturated to saturated fatty acids), thus linking hypothermia-induced changes in membrane composition to hypothermia-induced cell cycle arrest. We show that p53 phosphorylation and activation in the commercially relevant CHO (Chinese-hamster ovary)-K1 cell line is mediated by the ATR–p53–p21 pathway and ATR signalling is thus a key pathway involved in p53 activation upon mild hypothermia, and, furthermore, that cold-induced changes in cell membrane lipid composition are associated with this. We therefore provide the first mechanistic detail of cell sensing and signalling upon mild hypothermia in CHO cells leading to p53 and p21 activation, which are known to subsequently result in cell cycle arrest.

EXPERIMENTAL

Cells and cell maintenance

CHO-K1 cells (originally sourced from the European Collection of Cell Cultures) were maintained in DMEM (Dulbecco's modified Eagle's medium)/F12 (Invitrogen), supplemented with 10 % (v/v) dialysed heat-inactivated FBS (fetal bovine serum) (PAA, catalogue no. A15–507), glutamine, glutamate, aspartate, nucleosides and non-essential amino acids (Invitrogen), at 37 °C in a 5 % CO_2 atmosphere as described previously [12]. HeLa (Ohio) cells (sourced from the A.T.C.C.) were maintained in DMEM supplemented with 10 % (v/v) FBS (PAA, catalogue no. A15–151), 2 mM glutamine and non-essential amino acids. ^{35}S-labelled amino acid incorporation into proteins was assessed as described in [12]. Exposure to mildly sub-physiological temperatures was undertaken in routine culture medium in appropriately regulated (±0.1 °C) incubators. Exposure to 15 μM BEL (Sigma) was for 6 h at 37 °C in normal growth medium. Caffeine (Sigma) was used at a final concentration of 2.5 mM, wortmannin (Sigma) at a final concentration of 20 μM, the p38 kinase inhibitor SP203580 (Calbiochem) at a final concentration of 10 μM, the ATM (ataxia telangiectasia mutated) inhibitor KU0055933 at a final concentration of 10 μM and the DNA-PK (DNA-dependent protein kinase) inhibitor NU7441 at a final concentration of 1 μM. Cells were exposed to these inhibitors for 30 min at 37 °C prior to transfer, without removal of the inhibitor, to mildly hypothermic conditions. For RNAi (RNA interference) knockdown, cells were transfected with validated siRNAs (small interfering RNAs) for Hs ATR (*Homo sapiens* ATR), *Hs_ATR_11* (Qiagen) and *Hs_ATR_12* (Qiagen), using HiPerfect reagent (Qiagen) as described in the manufacturer's instructions and a final siRNA concentration of 5 nM. When combining RNAi knockdown of ATR with inhibitor experiments, CHO-K1 cells were first exposed to ATR siRNA for 48 h at 37 °C, then 10 μM SP203580 was added for a further 30 min prior to transfer, in the continued presence of siRNA and SP203580, to 32 °C or 27 °C for 10 h.

Extraction of RNA, protein and lipids from cells

Total RNA was extracted from intact cells using the commercially available Qiagen RNeasy kit as per the manufacturer's instructions. Cell lysates for protein analysis were prepared by lysing PBS-washed cells into ice-cold extraction buffer, 20 mM Hepes/NaOH, pH 7.2, containing 100 mM NaCl, 1 % (w/v) Triton X-100, protease inhibitors (10 μg/ml leupeptin, 2 μg/ml pepstatin and 0.2 mM PMSF) and protein phosphatase inhibitors (50 mM NaF and 1 mM activated Na_3VO_4). For each lipid extraction, 5×10^6 CHO-K1 cells (60 % confluent) were washed with 10 ml of 0.8 % NaCl at the appropriate temperature and then scraped into 600 μl of solid-CO_2-chilled methanol, followed by extraction of lipids by vortex-mixing for 15 s with 600 μl of solid-CO_2-chilled chloroform and then freezing in liquid N_2 for 1 min before thawing on ice. This freeze–thaw procedure was repeated twice more. The chloroform extract was then washed twice by adding 900 μl of ice-cold water, vortex-mixing for 30 s followed by centrifugation at 16 000 ***g*** for 15 min and removal of the aqueous layer. Samples were stored at −80 °C prior to transportation (as solutions) on solid CO_2 to Manchester for lipid analysis.

qRT-PCR (quantitative real-time PCR) analysis of mRNA levels

Relative quantification of mRNA levels was undertaken by qRT-PCR using the Bio-Rad Laboratories iScript™ one-step kit as described in the manufacturer's instructions with the following primers: human ATR, Quantitect primer assay Hs_ATR_1_SG (Qiagen); CHO ATR, forward, 5′-GTTAATCCATGGTCGAGC-3′, reverse, 5′-TTGTCATAGTACTTGGCAAGG-3′; human actin, forward, 5′-CCGAGGACTTTGATTGCAC-3′, reverse, 5′-AGTGGGGTGGCTTTTAGGAT-3′; and CHO actin, forward, 5′-AGCTGAGAGGGAAATTGTGCG-3′, reverse, 5′-GCAACGGAACCGCTCATT-3′. Reactions were carried out using a Mastercycler® ep Realplex thermocycler (Eppendorf) programmed for a reverse transcription incubation at 50 °C for 10 min, followed by a 95 °C hold for 5 min and subsequent 40 cycles of 10 s at 95 °C and 20 s at 55 °C.

SDS/PAGE and immunoblot analysis

For SDS/PAGE analysis, 10 % separation gels were prepared as described by Laemmli [21], loading 20 μg of protein lysate per lane. SDS/PAGE-resolved polypeptides were transferred on to a nitrocellulose membrane, which was then blocked with 5 % (w/v) non-fat dried skimmed milk powder in 0.2 % Tween 20/TBS (Tris-buffered saline). Primary antibodies were sourced as follows: anti-ATR, Santa Cruz Biotechnology (N19); anti-p53, Dako (clone DO-7); anti-p53 phosphorylated at Ser^{15}, Cell Signaling Technology (#9284); anti-p21, Santa Cruz Biotechnology (C19); and anti-β-actin, Sigma (clone AC15). Horseradish peroxidase-conjugated secondary antibodies were detected by enhanced chemiluminescence using Hyperfilm ECL (GE Healthcare). Linearity of the antibody response over the

concentration range of the target protein had been established previously [12].

Immunofluorescence microscopy

PBS-washed CHO-K1 cells grown on 13 mm glass coverslips were fixed in 3 % (w/v) paraformaldehyde in PBS for 15 min at 37 °C or 27 °C, then permeabilized with 0.5 % Triton X-100 in PBS for 10 min at room temperature (21 °C). After blocking in 0.1 % Tween 20/PBS containing 3 % (w/v) BSA, coverslips were incubated with anti-ATR antibody (1:50 dilution) overnight at 4 °C. Further processing and detection were as described in [12].

MS analysis of lipids

Profiling of the lipid fraction of cell extracts was performed using DIMS (direct infusion MS [22]). Chloroform extracts (400 μl) were diluted in 600 μl of methanol. Samples were infused into an electrospray Thermo Fisher Scientific LTQ-Orbitrap XL mass spectrometer operating in negative-ion mode at a flow rate of 5 $\mu l \cdot min^{-1}$ for 1 min. Accurate mass data were acquired in the Orbitrap mass analyser operating at a mass resolution of 100000 (at *m/z* 400) and with a scan time of 1.2 s. All mass spectra were averaged to provide a single mass spectrum for each sample that was passed forward for further data processing and analysis. All mass peaks were binned to the nearest nominal mass (defined as mass bins).

Data analysis of lipid profiles

Multivariate PC-DFA (principal component-discriminant function analysis) using cross-validation was performed to inspect the clustering of sample classes as detailed in [23]. All data were normalized to a sum of 1. A PCA (principal component analysis) model was then constructed [24] with three of six samples per class; the first ten PCs (principal components) were extracted and these accounted for 99.8 % of the total explained variance. Next, these PCs were used by the DFA algorithm [25] with the *a priori* knowledge of the six different treatments. In order to validate this PC-DFA model, it was tested by cross-validation by the projection of the three remaining samples, a process that allows cluster integrity to be assessed; that is to say, if the projected samples co-cluster with samples used to construct the PC-DFA model, then the groupings observed and their relationships are valid. All multivariate analyses were performed in MatLab (MathWorks). To define statistically significant differences, Kruskal–Wallis analysis of variance was performed. For those mass bins of statistical significance, further analysis was performed to determine the accurate lipid mass contributing to this statistical difference. Putative identification of lipids (as the deprotonated ion, sodiated or potassiated negatively charged adducts) was performed by matching to the accurate mass of lipids in the Lipid Maps database (http://www.lipidmaps.org/) with a mass accuracy less than 2 p.p.m.

RESULTS

p53 is phosphorylated at Ser^{15} when CHO-K1 cells are exposed to mildly hypothermic conditions

When CHO-K1 cells were transferred from 37 °C to 32 °C the subsequent growth rate was greatly reduced, and cells transferred to 27 °C ceased to proliferate (Figure 1A). Under both of these mildly hypothermic conditions, expression of p21 was induced and maintained throughout a 5 day period, whereas phosphorylation of p53 at Ser^{15} initially increased dramatically, but then decreased towards the end of the 5 day period (Figure 1B). Notably, the levels of both p53 phosphorylation and p21 expression were greater at 27 °C compared with 32 °C in line with the proliferation status of the cells at these two temperatures, and at 32 °C cell proliferation was observed towards the end of the 5 day period when Ser^{15} phosphorylation levels once again decreased (Figure 1B). A more detailed examination of the early period following the temperature shift to 27 °C (Figure 1C) clearly showed that the phosphorylation of p53 at Ser^{15} preceded a modest increase in p53 levels that, in turn, preceded the induction of p21. This

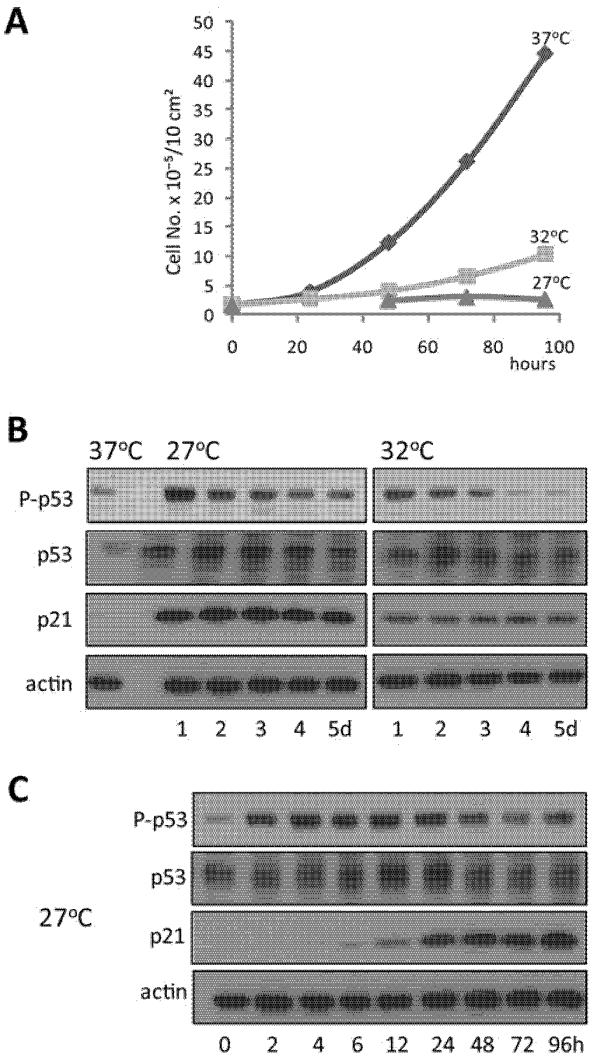

Figure 1 Mild hypothermia reduces cell proliferation and induces phosphorylation of p53 at Ser^{15} and subsequent p21 expression

(**A**) Growth curves of CHO-K1 cells maintained at the indicated temperatures. (**B**) Immunoblot detection of p53 phosphorylated at Ser^{15}, total p53 protein and p21 in lysates of CHO-K1 cells maintained at the indicated temperatures for the indicated number of days. (**C**) Immunoblot detection of p53 phosphorylated at Ser^{15}, total p53 protein and p21 in lysates of CHO-K1 cells maintained at 27 °C for the indicated number of hours. In (**B** and **C**), immunoblot detection of β-actin served as an indicator of lysate protein loading.

is consistent with the response to hypothermia being due to stabilization of p53 consequent to its phosphorylation at Ser^{15} and this increased level of p53 then inducing p21 expression.

The ATR protein kinase regulates phosphorylation of p53 at Ser^{15} upon exposure to mild hypothermia

Having established that p53 is phosphorylated at Ser^{15} in response to mild hypothermia, we set out to establish the kinase(s) responsible for this phosphorylation. Phosphorylation at Ser^{15} of p53 can be mediated by several protein kinases, including ATM, ATR, DNA-PK and the stress response signalling pathway protein kinase $p38^{MAPK}$ (p38 mitogen-activated protein kinase) [26]. To determine whether any of these was effecting p53 phosphorylation during mild hypothermia, we used a combination of general and specific protein kinase inhibitors and siRNA knockdown. Initially, we used caffeine, a well known, although not very specific, inhibitor of the PIKK family of protein kinases [27]. In the concentration range usually employed (low millimolar) it inhibits both ATM and ATR, but DNA-PK is relatively resistant. However, another PIKK family member, mTOR (mammalian target of rapamycin), a protein kinase that positively regulates protein synthesis in response to nutrient availability and growth factor signalling, is also inhibited by low millimolar concentrations of caffeine [27]. This must be taken into account when assessing the effect of caffeine on hypothermia-induced p21 expression. In the short term, 2.5 mM caffeine inhibited phosphorylation of p53 at Ser^{15} when cells were transferred to 32 °C, but had little effect when cells were transferred to 27 °C (Figure 2A). During longer-term exposure to caffeine, phosphorylation of p53 at Ser^{15} was less sustained than in the absence of caffeine and p21 expression was reduced, under both hypothermic conditions investigated (32 and 27 °C) (Figure 2B). When compared with the inhibition of general protein synthesis by caffeine (due to mTOR inhibition), the inhibition by caffeine of p21 expression was greater (Figure 2D), consistent with either ATM or ATR being involved in hypothermia-induced expression of p21. More specific inhibition of DNA-PK with NU7441 [28] had no effect on either hypothermia-induced phosphorylation of p53 at Ser^{15} or induction of p21 (Figures 3A and 3B). Thus, of the potential PIKK kinases that could phosphorylate p53 at Ser^{15} upon mild hypothermia, these results suggested that either ATM or ATR is responsible.

The fungal metabolite wortmannin is a widely used, irreversible, inhibitor of phosphoinositide 3-kinases, and treatment of cells with micromolar concentrations of this compound causes inhibition of ATM, DNA-PK and mTOR [29]. However, ATR is relatively resistant to wortmannin, and cells require exposure to concentrations in excess of 100 μM before ATR is inhibited [29]. In agreement with the results from the caffeine studies, which suggested that ATR might phosphorylate p53 at Ser^{15}, 20 μM wortmannin had no effect on hypothermia-associated phosphorylation of p53 at Ser^{15} and marginally inhibited p21 induction (Figure 2C). However, in contrast with inhibition by caffeine, inhibition of general protein synthesis by wortmannin was not significantly different from inhibition of hypothermia-induced p21 expression by wortmannin (Figure 2E). We then used a specific inhibitor of ATM, KU0055933 [28], and this inhibited neither hypothermia-associated phosphorylation of p53 at Ser^{15} nor induction of p21 (Figures 3A and 3B). Therefore, using specific inhibitors to DNA-PK and ATM, we were able to demonstrate that neither is the primary kinase involved in the hypothermia-induced p53–p21 pathway.

Although these inhibitor data are consistent with a signalling pathway in which ATR is a key kinase in the hypothermia-induced p53–p21 pathway, they are not specific ATR inhibitors, therefore, to test this hypothesis further, siRNA knockdown of *ATR* mRNA was employed. This approach has been shown to effectively reduce ATR protein levels by approx. 70 % 24 h after transfection [30,31] and therefore, although this does not obliterate protein levels, a knockdown would be expected to result in decreased Ser^{15}-phosphorylated p53 in response to mild hypothermia if this kinase is responsible. Two commercial validated siRNAs to human ATR were tested for their ability to knock down CHO-K1 *ATR* mRNA due to the lack of availability of such reagents for CHO ATR. As expected, both siRNAs efficiently decreased HeLa cell *ATR* mRNA over a 48 h period by between 67 and 77 % (Figure 4A). When tested in CHO-K1 cells, exposure to one of these siRNAs for 48 h decreased CHO *ATR* mRNA by 77–87 %. However, knockdown by the second siRNA was less effective and more variable in CHO cells (Figure 4A). Knockdown of ATR mRNA was maintained at 72 h and, to a lesser degree, at 96 h post-transfection (Figure 4A). We confirmed that knockdown of *ATR* mRNA resulted in a knockdown in ATR protein levels in both HeLa and CHO cells by Western blotting, which showed that ATR protein levels were reduced by 55–85 % after a 48 h exposure to ATR siRNA (Figure 4B). Following transfection with these siRNAs, cells were maintained at 37 °C for 48 h before transfer to either 32 °C or 27 °C for a further 10 h. The decreases in ATR mRNA and protein observed after a 48 h exposure to ATR siRNA were clearly mirrored by the decrease in the extent of phosphorylation at Ser^{15} of p53 under these mildly hypothermic conditions (Figure 4C). Inhibition of cold-induced phosphorylation of p53 at Ser^{15} was still evident at 72 h, but not at 96 h, post-transfection (Figure 4D), but at this last time point the hypothermia-associated phosphorylation of p53 was already in decline (Figures 1B and 4D). These results are consistent with the inhibitor data indicating that hypothermia induces p53 phosphorylation and p21 activation through the ATR–p53–p21 signalling pathway. Furthermore, the relative longevity (several days) of p53 phosphorylation at Ser^{15} during hypothermia is also consistent with this phosphorylation being regulated by ATR [32]. We note that although knockdown of ATR protein was clearly achieved, ATR protein was still present and some phosphorylated p53 was also present in the knockdown experiments (Figures 4C and 4D). We were unable to ascertain from these results whether the phosphorylated p53 present upon cold shock was due to the residual ATR protein present or as a result of an additional pathway not investigated in the present study. Despite this, when cells were shifted to 27 °C following knockdown of ATR for 48 h at 37 °C by siRNA, those wells in which knockdown had been undertaken initially showed an increased in cell numbers 1 and 2 days after being placed at 27 °C above that observed in the mock knockdown (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/435/bj4350499add.htm). This further suggests that p53 activation through ATR is involved in the inhibition of cell proliferation upon cold shock at 27 °C. This effect was lost after 2 days at 27 °C, probably because, at this stage, the knockdown cells at a higher cell concentration are beginning to experience nutrient and growth stresses that lead to a decrease in cell number as seen in Supplementary Figure S1.

Involvement of the $p38^{MAPK}$ stress kinase signalling pathway in cell cycle arrest during mild hypothermia

Although our results show that ATR is involved in the regulation of p53 Ser^{15} phosphorylation upon mild hypothermia and rule out

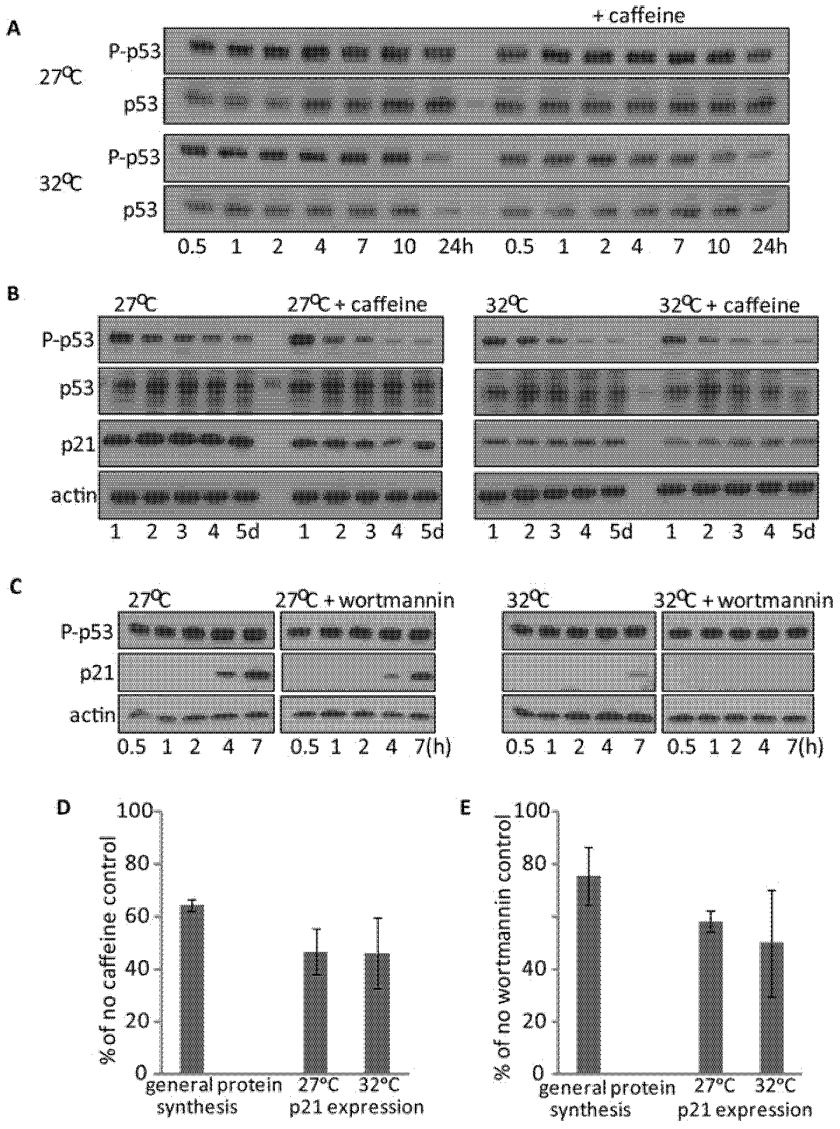

Figure 2 Caffeine inhibits both phosphorylation of p53 at Ser[15] and p21 induction associated with mild hypothermia, but wortmannin does not

(**A**) Immunoblot detection of p53 phosphorylated at Ser[15] and total p53 protein in lysates of CHO-K1 cells, with or without pre-treatment with 2.5 mM caffeine for 30 min at 37 °C immediately prior to exposure to the indicated temperatures for the indicated number of hours. (**B**) Immunoblot detection of p53 phosphorylated at Ser[15], total p53 protein, p21 and β-actin in lysates of CHO-K1 cells, with or without pre-treatment with 2.5 mM caffeine for 30 min at 37 °C immediately prior to exposure to the indicated temperatures for the indicated number of days. (**C**) Immunoblot detection of p53 phosphorylated at Ser[15], p21 and β-actin in lysates of CHO-K1 cells exposed to 20 μM wortmannin for 30 min at 37 °C prior to incubation for the indicated times at 27 °C or 32 °C. The response of total p53 protein levels for these time points at 27 °C and 32 °C are shown in (**B**). (**D**) Quantification of the inhibition by 2.5 mM caffeine of general protein synthesis and of hypothermia-induced p21 expression. (**E**) Quantification of the inhibition by 20 μM wortmannin of general protein synthesis and of hypothermia-induced p21 expression.

ATM and DNA-PK, this phosphorylation could also be effected by the stress response protein kinase p38^{MAPK} (Hog1 in yeast), either directly [33] or through its phosphorylation at Ser[33] and Ser[46] of p53 that, in turn, enhances phosphorylation at Ser[15] [34]. In yeast, this protein kinase is activated by osmotic stress or exposure to cold [35], whereas in mammalian cells, it has also been shown to be activated by osmotic stress [36]. p38^{MAPK} is also activated by hypoxia and it has been reported that mildly hypothermic mammalian cells are hypoxic [37]. Furthermore, ATR can also phosphorylate, and thereby activate, p38^{MAPK} [38]. It was therefore considered important to determine whether the p38^{MAPK} protein kinase was involved, either independently, or through activation by ATR, in the p53–p21 pathway induced by mild hypothermia.

SP203580 is an inhibitor frequently used for assessing involvement of p38^{MAPK} in signalling pathways [39]. Although this inhibitor can also inhibit casein kinase 1 [5], this kinase will not phosphorylate p53 at Ser[15] [40], therefore this inhibitor allowed us to investigate potential p38^{MAPK} involvement in hypothermia-induced phosphorylation of p53 Ser[15]. Treatment of CHO-K1 cells with 10 μM SP203580 for 30 min prior to transfer to 27 °C or 32 °C reduced both phosphorylation at Ser[15] of p53 and expression of p21 at these temperatures (Figure 5A). Since

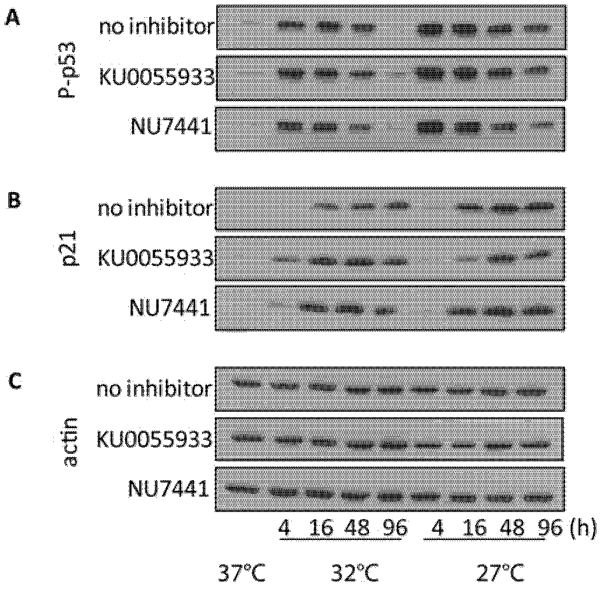

Figure 3 Specific inhibitors of DNA-PK and ATM do not abrogate the cold-induced phosphorylation of p53 and induction of p21

CHO-K1 cells were grown at 37 °C for 24 h, then 1 μM DNA-PK inhibitor NU7441 or 10 μM ATM inhibitor KU0055933 was added as indicated. After a further 30 min of incubation at 37 °C, cells were either maintained at 37 °C or transferred to 32 °C or 27 °C for the indicated number of hours. Immunoblots of cell lysates were probed for Ser^{15}-phosphorylated p53 (**A**), p21 (**B**) and β-actin (**C**). Total p53 protein levels at these temperatures (4, 48 and 96 h) were established previously and reported in Figures 1 and 2.

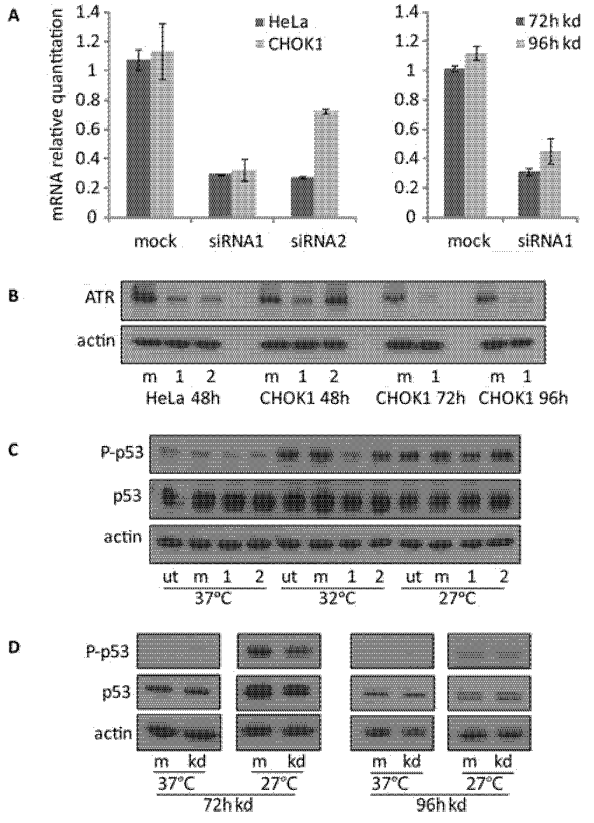

Figure 4 siRNA knockdown of ATR mRNA inhibits hypothermia-induced phosphorylation of p53 at Ser^{15}

(**A**) HeLa and CHO-K1 cells were transfected with 5 nM siRNAs against human *ATR* mRNA and then maintained at 37 °C for 48–96 h before undertaking qRT-PCR analysis of *ATR* mRNA levels in total RNA. (**B**) Immunoblot detection of ATR in HeLa and CHO-K1 cell lysates prepared after 48–96 h of exposure to ATR siRNAs at 37 °C (m, mock transfected; 1, ATR siRNA 1; 2, ATR siRNA 2). (**C**) Immunoblot detection of total p53 protein and p53 phosphorylated at Ser^{15} in cell lysates of CHO-K1 cells 48 h after siRNA knockdown of *ATR* mRNA at 37 °C followed by 10 h at 27 °C or 32 °C (ut, untreated; m, mock transfected; 1, ATR siRNA 1; 2, ATR siRNA 2). (**D**) Inhibition of phosphorylation of p53 at Ser^{15} is maintained over longer periods of siRNA knockdown (kd) of ATR than 48 h. CHO-K1 cells were transfected with siRNA 1 or mock transfected (m), incubated at 37 °C for 72 h or 96 h and then maintained at 37 °C or transferred to 27 °C for a further 10 h prior to extraction for immunoblot detection of the indicated proteins.

SP203580 had no effect on general protein synthesis (Figure 5B), its inhibition of p21 expression suggested involvement of p38^{MAPK} in the hypothermia-induced p53–p21 pathway.

To determine whether this p38 mechanism was a second pathway leading to phosphorylation at Ser^{15} of p53 independently of the ATR route, treatment with SP203580 was combined with siRNA knockdown of ATR. The resulting effects of combined ATR knockdown and SP203580 treatment on hypothermia-induced phosphorylation at Ser^{15} of p53 and the p53 isoform pattern (Figure 6) mirrored those effects observed for ATR knockdown alone (Figure 4). This suggests that the involvement of p38^{MAPK} in hypothermia-induced cell cycle arrest lies within, rather than acts independently of, the ATR pathway; otherwise, the effects of ATR knockdown and SP203580 treatment should have been additive. Therefore we suggest that the p38^{MAPK} protein kinase is involved in phosphorylation of p53 at Ser^{15} upon mild hypothermia through activation by ATR.

How is ATR activated upon exposure of CHO-K1 cells to mild hypothermia?

Our results confirmed that ATR is activated upon CHO-K1 cells being exposed to mild hypothermia, which in turn phosphorylates Ser^{15} of p53 and p21 induction. However, how might ATR itself be activated upon mild hypothermia? We used immunofluorescence to determine whether there was any change in the localization of ATR following cold shock (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/435/bj4350499add.htm). Using this approach, it was found that at 2–48 h after cold shock at 27 °C, ATR appeared to be concentrated into the nucleolus (Supplementary Figure S2). We also noted an overall increase in ATR-associated fluorescence throughout the cell, particularly between 6 and 24 h of exposure to 27 °C.

In addition to localization studies, we investigated changes to the lipid content of cold-shocked cells. When prokaryotic and lower eukaryotic cells are exposed to hypothermic conditions, the unsaturated fatty acyl content of cell membrane lipids has been reported to increase [14]. In mammalian cells, exposure to the Ca^{2+}-independent phospholipase A_2 inhibitor BEL at 37 °C also increases the unsaturated fatty acyl content of phosphatidylcholines and activates ATR [17]. We therefore compared the effect of BEL treatment with that of hypothermia on cellular lipid composition to determine whether a similar effect could be observed that might offer an explanation of ATR activation upon mild hypothermia. To achieve this, MS analysis of total lipids extracted from cells maintained at normal temperature (37 °C), after treatment with BEL, and at mildly hypothermic temperatures was performed. Multivariate analysis (PC-DFA) was applied to the resulting data with cross-validation as described

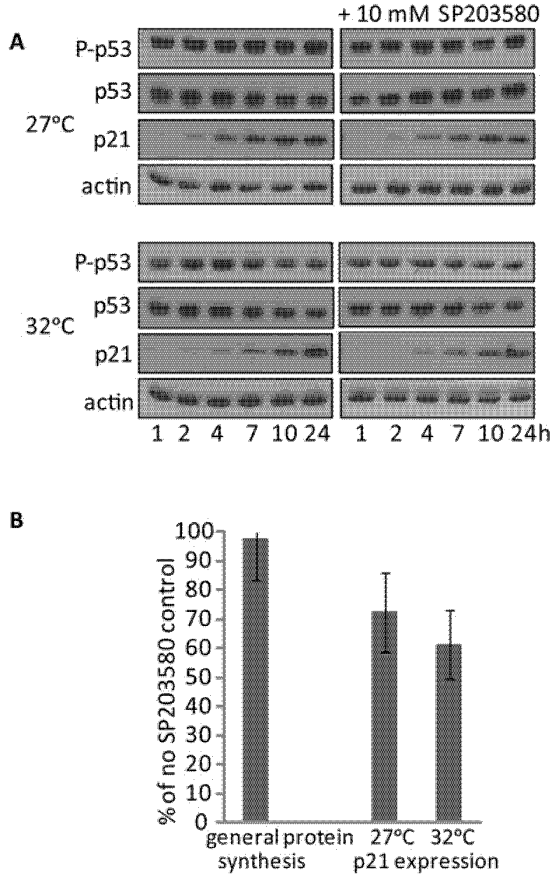

Figure 5 The p38^{MAPK} inhibitor SP203580 attenuates hypothermia-associated phosphorylation of p53 at Ser^{15} and p21 induction

(**A**) CHO-K1 cells were exposed to 10 μM SP203580 for 30 min at 37 °C and then transferred to 27 °C or 32 °C for the indicated times (1–24 h). Immunoblot detection of p53 phosphorylated at Ser^{15}, total p53 protein and p21 with β-actin as an indicator of protein loading is shown. (**B**) Quantification of the effects of 10 μM SP203580 on general protein synthesis and hypothermia-induced p21 expression.

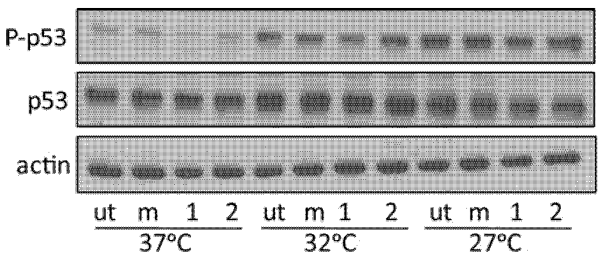

Figure 6 Inhibition of hypothermia-induced phosphorylation of p53 at Ser^{15} by ATR knockdown is not increased by additional inhibition of p38^{MAPK}

At 48 h after siRNA-mediated *ATR* mRNA knockdown, CHO-K1 cells were additionally exposed to SP203580 for 30 min at 37 °C then transferred to 27 °C or 32 °C for a further 10 h. Immunoblot detection of total p53 protein, p53 phosphorylated at Ser^{15} and β-actin in lysates of CHO-K1 cells treated in this way is shown. ut, untreated; m, mock transfected; 1, ATR siRNA 1; 2, ATR siRNA 2 as in Figure 4.

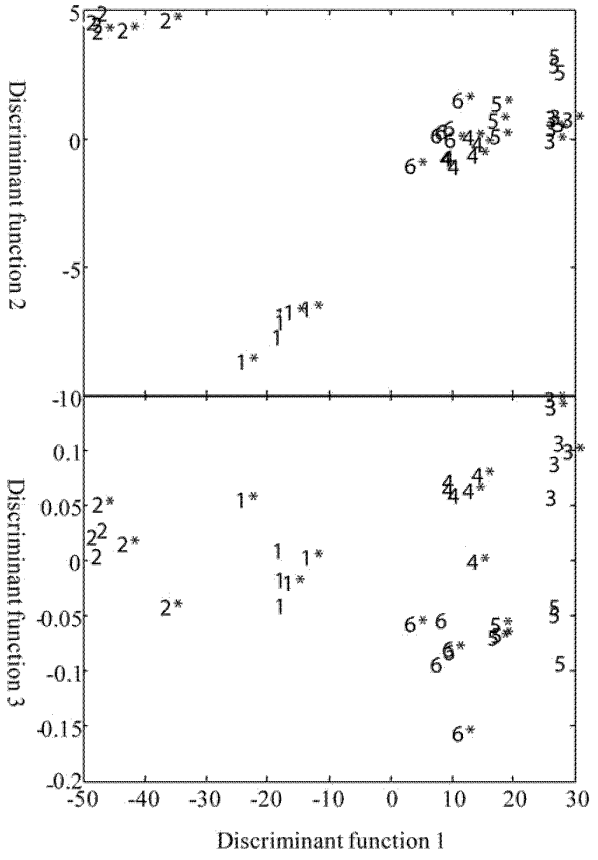

Figure 7 Exposure of CHO-K1 cells to mild hypothermia is associated with changes in the cellular lipid profile

PC-DFA of all samples. Upper panel: PC-DF1 plotted against PC-DF2. Lower panel: PC-DF1 plotted against PC-DF3. The first ten PCs were used by the DFA algorithm and this accounted for 99.8% of the total explained variance. The multivariate model was constructed using three of six samples in each class (no asterisk) and cross-validated by projection of the remaining three samples (shown with an asterisk). The level of agreement of the samples projected with those used to construct the model highlight that the model is validated. Class 1, control maintained at 37 °C for 6 h with no treatment; Class 2, control maintained at 37 °C for 6 h with BEL treatment; Class 3, maintained at 27 °C for 6 h; Class 4, maintained at 32 °C for 6 h; Class 5, recovery at 37 °C for 2 h after a temperature of 27 °C for 6 h; Class 6, recovery at 37 °C for 2 h after a temperature of 32 °C for 6 h.

in the Experimental section and shown in Figure 7. The results show that BEL-treated and 37 °C control cells were different from all cells cultured at reduced temperatures, and the chemical treatment and control cells dominated the separation of the second canonical variate (Figure 7, upper panel). When PC-DF1 was plotted against PC-DF3 (Figure 7, lower panel), each class was biologically distinct from other classes, highlighting the fact that the detectable lipid profile of each of the six classes was different and perturbations (chemical or temperature-based) resulted in distinct phenotypic changes.

Furthermore, univariate analyses using Kruskal–Wallis analysis of variance to define the lipids that were statistically significantly changing (see Supplementary Table S1 at http://www.BiochemJ.org/bj/435/bj4350499add.htm) revealed that the positive control (treatment with BEL) showed a different relative change to the control in the PC-DFA model when compared with the temperature-treated cells. Ten lipids were statistically different ($P < 0.05$) and all showed an increase in

their relative concentration in the BEL-treated cells compared with the control. Cells treated at 27 °C and 32 °C (mild hypothermia) for 6 h showed a similar trajectory from the control samples, with the 27 °C samples showing a greater biological difference in multivariate space compared with the samples perturbed at 32 °C. However, more statistically significant changes were observed between control and 32 °C samples in the univariate analysis (37 compared with four changes for 32 °C and 27 °C respectively). All of the changes showed an increase in concentration of a range of lipids, predominantly phospholipids (diacylglyceroserines, diacylglyceroinositols and diacylglycerophosphocholines). In most cases, although not exclusively, an increase in the unsaturated double-bond content was present in the lipids of increased abundance. This highlights a definitive increase in the production of a specific class of lipids in response to temperature-based perturbations. The increase in temperature after hypothermia perturbation (recovery) provides a change in the lipid profile from that at reduced temperature, but this lipid profile is distinct from all other samples. This shows that an increase in temperature changes the lipid profile, but not to a normal profile at 2 h after the return to 37 °C. Decreases in the relative concentration of lipids were observed in the change from lower to higher temperature, of the same classes of lipids that were observed to increase as the temperature was decreased. This highlights the specific role of these lipids in the response to temperature perturbation and how their relative concentration is temperature dependent. Although many of the lipids were chemically identified, we were unable to show significant changes in the overall unsaturated fatty acyl content of cell membrane lipids. However, we have shown an increase in polyunsaturated lipids upon mild hypothermia consistent with a previous study showing that an increase in phosphatidylcholines containing polyunsaturated fatty acids activates ATR–p53 signalling at 37 °C [17].

DISCUSSION

Although we [12] and others [10,13] have documented that p53 activation of p21 is a key mechanism by which mammalian cells initiate cell cycle arrest upon being subjected to mild hypothermic temperatures, the mechanism by which p53 is activated and the cellular mechanisms that allow the perception of cold and subsequent activation of p53 have remained undetermined. In the present study, we have shown that the exposure of CHO-K1 cells to mildly hypothermic conditions activates the ATR kinase that subsequently activates p53 by phosphorylation at Ser^{15} and hence the ATR–p53–p21 signalling pathway. We note that although our experiments clearly show ATR regulation of p53 phosphorylation upon cold shock, in our ATR knockdown and inhibitor experiments some ATR protein and phosphorylated p53 still remained and we were unable to ascertain from these results whether the phosphorylated p53 present upon cold shock in these experiments was due to the residual ATR protein present or a result of an additional signalling pathway not investigated in the present work.

We speculate that the primary stimulus for the activation of the ATR–p53–p21 signalling pathway upon mild hypothermia may be changes in membrane rigidity [14] as a direct result of changes in membrane lipid composition (homeoviscous adaptation). Our results show changes in the levels of polyunsaturated fatty acids upon cold shock that are known to influence the fluidity of cellular membranes, and, furthermore, that these changes correlated with the activation of ATR. As described above, a previous study has demonstrated that changes to cell membrane fluidity and increased polyunsaturation activates ATR and the authors of that study suggest that this occurs as a result of ATR 'sensing' the change in the ratio of polyunsaturated to saturated hydrocarbons [17]. The question is how might this change in lipid composition activate ATR? Zhang et al. [17] suggest that this is the result of changes in the fluidity and function of the nuclear envelope whereby the nuclear-localized ATR senses these changes and is activated. We speculate further that this leads to an intranuclear relocalization of ATR upon activation (as shown in Supplementary Figure S2), p53 activation and cell cycle arrest. Such intranuclear relocalization of ATR to nuclear foci has been documented in response to both hypoxia [41] and DNA damage [42]. The overall increase in ATR-associated fluorescence throughout the cell during early exposure to 27 °C without an increase in immunoblot detection of ATR also suggests that, additionally, there may be a conformational change in ATR upon exposure of the cell to cold that renders the protein more accessible to the anti-ATR antibody used.

CHO-K1 p53 carries a single point mutation at codon 211 in exon 6 in the DNA-binding domain of the molecule, although this mutation is not within an evolutionarily conserved region [43]. Furthermore, CHO-K1 p53 is rather more abundant and stable than wild-type p53. At 37 °C, its half-life is 5.2 h [12] compared with the more usual range of 20–60 min for p53 half-lives. Furthermore, CHO-K1 p53 is not stabilized further, and thereby increased in amount, by ionizing radiation, i.e. by the ATM signalling pathway alone [43]. Thus, even though CHO-K1 p53 is relatively abundant, it is not sufficient, under normal conditions, to activate transcription of p21. Even under mildly hypothermic conditions, when p21 transcription is activated, increases in CHO-K1 p53 total protein are very modest (Figures 1, 2, 4 and 6). What does change markedly in response to hypothermia is the phosphorylation status of p53. For wild-type p53, phosphorylation at Ser^{15} enhances p53 transactivation of p21 transcription by increasing the binding of p53 to its transcriptional co-activator, p300/CBP [44]. Furthermore, although phosphorylation at Ser^{15} of p53 is not itself sufficient to disrupt the interaction between p53 and Mdm2 (murine double minute 2) that targets p53 for degradation, phosphorylation at this site is a prerequisite for phosphorylation at Ser^{20} of p53. Ser^{20} phosphorylation inhibits the binding of p53 to Mdm2 [45]. The overall effect of phosphorylation of Ser^{15} of wild-type p53 is therefore 2-fold, i.e. enhanced stability and enhanced transcriptional activation ability. In the context of CHO-K1 cells, this must mean that phosphorylation at Ser^{15} is sufficient to enhance the transcription factor activity of an already abundant p53, even though this transactivation activity might be compromised to some extent by the point mutation in the DNA-binding domain of CHO-K1 p53.

A consistent finding that has emerged from the numerous studies of p53 post-translational modifications is that phosphorylation and acetylation sites are seldom modified alone and that post-translational modification at one site is often a prerequisite for further post-translational modifications elsewhere on p53 [26]. This activation of p53 at more than one site has been termed 'intramolecular phosphorylation site interdependence' [46] and is nearly always required before downstream transcriptional activation takes place. These results suggest that p53 transcriptional activation is tightly regulated by multiple modifications, thus minimizing inappropriate transcriptional activation by p53 and providing a point of integration of signals from multiple protein kinases [46]. This appears to be the case for activation of p53 by mild hypothermia, since we have found that $p38^{MAPK}$ is also involved in the ATR–p53–p21 pathway.

Although it is well established that ATR directly phosphorylates p53 at Ser^{15}, there are conflicting reports regarding the ability of

p38^{MAPK} to directly phosphorylate p53 at Ser^{15} [33,34]. However, some of the transient transfection experiments used to delineate this may have been complicated by the transfection vehicle itself eliciting a stress response involving phosphorylation of p53 and activation of p21 transcription [34]. We too have noted this effect of some transfection reagents on p53 activation (A. Roobol and C. M. Smales, unpublished work) and suggest that this, and indeed the induction of p21 expression by addition of DMSO to the culture medium mentioned earlier, may be consequent to changes in membrane fluidity or composition. Nevertheless, it has been established that p38^{MAPK} phosphorylates p53 at Ser^{33} and Ser^{46} and that, when p53 is doubly phosphorylated at these two sites, phosphorylation at Ser^{15} by other protein kinases is enhanced [34]. It has also been shown that, in mammalian cells, activation of p38^{MAPK} by hypoxia is mediated by ATR [47].

These findings, when combined with the results we have presented here, suggest that mild hypothermia activates the transcription of p21 through ATR activation and subsequent phosphorylation of p53 at Ser^{15}. At the same time, we suggest that ATR activates p38^{MAPK}, resulting in the phosphorylation of p53 at Ser^{33} and Ser^{46} that subsequently enhances Ser^{15} phosphorylation. In this way, activated p53 subsequently activates transcription of the downstream target p21, and induction of p21 is known to lead to cell cycle arrest upon mild hypothermia.

AUTHOR CONTRIBUTION

Mark Smales and Anne Willis conceived the initial study. Mark Smales, Anne Willis, Anne Roobol, Jo Roobol, Amandine Bastide and Martin Carden devised the experimental plans. Anne Roobol and Jo Roobol carried out the majority of the experimental work. Warwick Dunn and Royston Goodacre devised, carried out and completed the lipid analysis. Anne Roobol, Mark Smales, Anne Willis, Warwick Dunn and Royston Goodacre wrote the manuscript. All authors analysed the results and read and approved the manuscript.

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online data

SUPPLEMENTARY ONLINE DATA

ATR (ataxia telangiectasia mutated- and Rad3-related kinase) is activated by mild hypothermia in mammalian cells and subsequently activates p53

Anne ROOBOL*, Jo ROOBOL*, Martin J. CARDEN*, Amandine BASTIDE†, Anne E. WILLIS†[1], Warwick DUNN‡, Royston GOODACRE‡ and C. Mark SMALES*[1]

*Centre for Molecular Processing and Protein Science Group, School of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, U.K., †MRC Toxicology Unit, Hodgkin Building, P.O. Box 138, University of Leicester, Lancaster Road, Leicester LE1 9HN, U.K., and ‡School of Chemistry and Manchester Centre for Integrative Systems Biology, Manchester Interdisciplinary Biocentre, University of Manchester, Manchester M1 7DN, U.K.

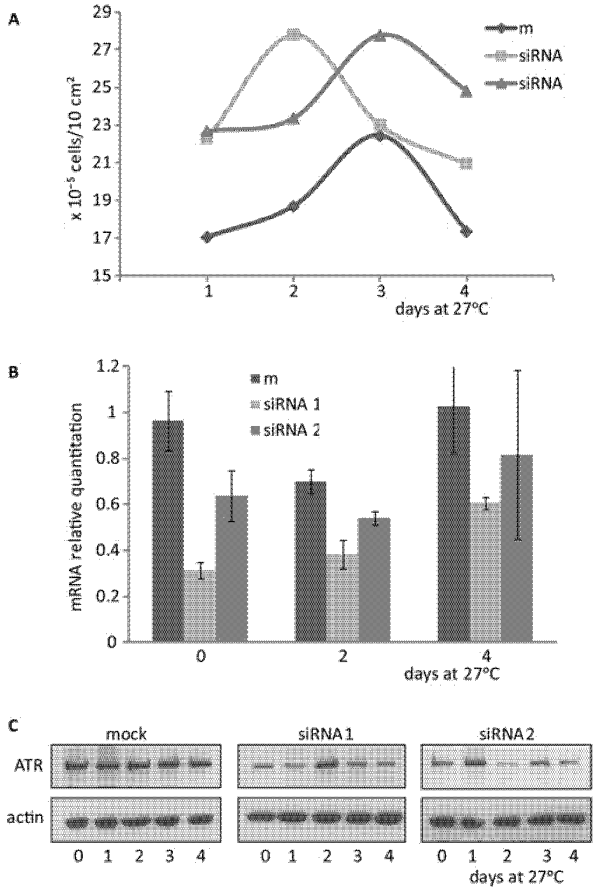

Figure S1 Effect of prolonged siRNA knockdown of ATR on cell proliferation at 27 °C

CHO-K1 cells were transfected with 5 nM ATR siRNA as described in the main text and then maintained at 37 °C for 48 h prior to transfer to 27 °C (day 0 on the indicated time scales). Samples were prepared on the indicated days of maintenance at 27 °C for cell counts (**A**), qRT-PCR quantification of *ATR* mRNA (**B**), and immunoblot detection of ATR protein (**C**). Mock transfections (m), siRNA1 and siRNA2 are as described in the Experimental section of the main text.

[1] Correspondence may be addressed to either of these authors (email aew5@le.ac.uk or c.m.smales@kent.ac.uk).

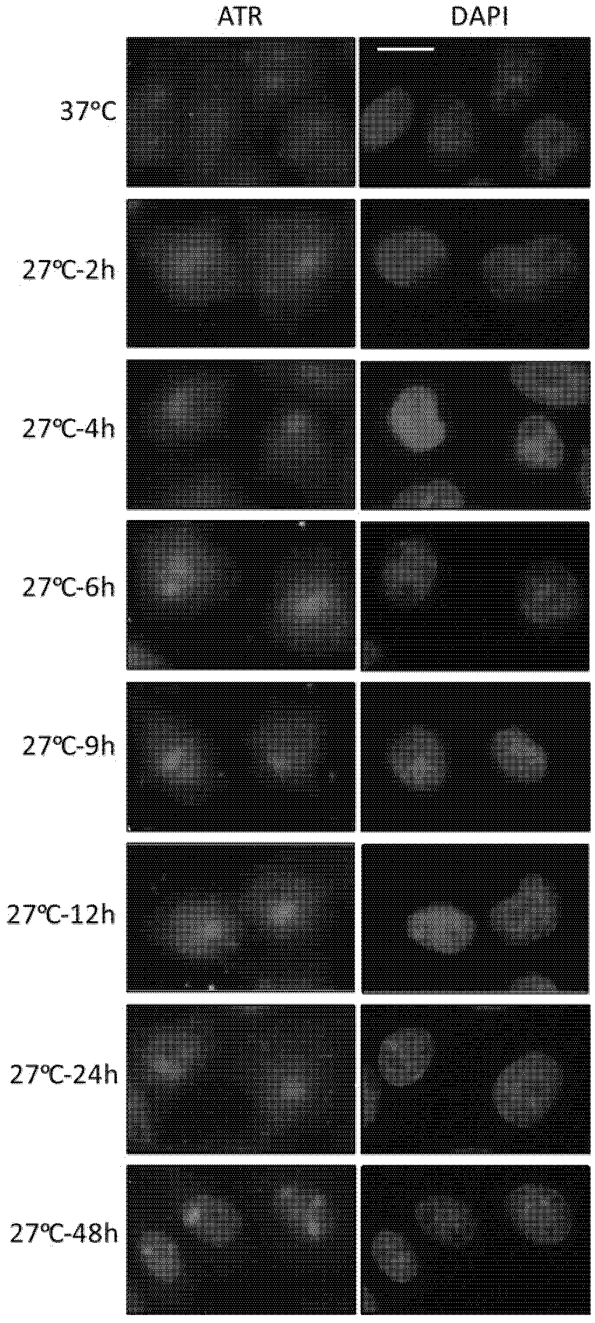

Figure S2 The intracellular localization of ATR changes during the early stages of hypothermia at 27 °C

Immunofluorescence detection of ATR and nuclei [visualized using DAPI (4′,6-diamidino-2-phenylindole)] in CHO-K1 cells maintained at 37 °C and during the first 48 h after transfer to 27 °C. Scale bar, 10 μm.

Table S1 Description of statistically significantly different metabolites

PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, prostaglandin; PI, phosphatidylinositol; PS, phosphatidylserine.

(a) Class 1 (control maintained at 37 °C for 6 h with no treatment) compared with Class 2 (control maintained at 37 °C for 6 h with BEL treatment).

P value	Fold difference	Lipid identification	Lipid class
0.00395	0.33 (lower in control)	PG(18:0/20:4)	Diacylglycerophosphoglycerols
0.00395	0.35 (lower in control)	PG(18:0/22:6)	Diacylglycerophosphoglycerols
0.00395	0.66 (lower in control)	Unidentified	Unidentified
0.00395	0.58 (lower in control)	PI(18:0/18:0)	Diacylglycerophosphoinositols
0.01041	0.66 (lower in control)	Unidentified	Unidentified
0.01041	0.43 (lower in control)	PG(17:0/17:0)	Diacylglycerophosphoglycerols
0.01631	0.73 (lower in control)	Unidentified	Unidentified
0.01631	0.70 (lower in control)	Unidentified	Unidentified
0.01631	0.76 (lower in control)	Unidentified	Unidentified
0.02498	0.69 (lower in control)	Unidentified	Unidentified

(b) Class 1 (control maintained at 37 °C for 6 h with no treatment) compared with Class 3 (maintained at 27 °C for 6 h).

P value	Fold difference	Lipid identification	Lipid class
0.00395	0.32 (lower in control)	Unidentified	Unidentified
0.00395	0.34 (lower in control)	PS(18:0/18:1)	Diacylglycerophosphoserines
0.01041	0.42 (lower in control)	PA(16:0/18:1)	Diacylglycerophosphates
0.02498	0.40 (lower in control)	Unidentified	Unidentified

(c) Class 1 (control maintained at 37 °C for 6 h with no treatment) compared with Class 4 (maintained at 32 °C for 6 h).

P value	Fold difference	Lipid identification	Lipid class
0.00395	5.88 higher in hypothermia	Unidentified	Unidentified
0.00395	2.08 higher in hypothermia	Unidentified	Unidentified
0.00395	2.86 higher in hypothermia	PS(18:0/18:1)	Diacylglycerophosphoserines
0.00395	3.22 higher in hypothermia	Unidentified	Unidentified
0.00395	1.82 higher in hypothermia	Unidentified	Unidentified
0.00395	2.13 higher in hypothermia	Unidentified	Unidentified
0.00395	2.33 higher in hypothermia	Unidentified	Unidentified
0.00395	2.22 higher in hypothermia	Unidentified	Unidentified
0.00395	2.50 higher in hypothermia	PI(18:0/18:0)	Diacylglycerophosphoinositols
0.00395	2.27 higher in hypothermia	Unidentified	Unidentified
0.00649	3.13 higher in hypothermia	PA(16:0/18:1)	Diacylglycerophosphates
0.00649	1.61 higher in hypothermia	Unidentified	Unidentified
0.00649	1.88 higher in hypothermia	Unidentified	Unidentified
0.01041	1.69 higher in hypothermia	PE(16:0/18:3)	Diacylglycerophosphoethanolamines
0.01041	1.96 higher in hypothermia	1-Tetrahexanoyl-2-(8-[3]-ladderane-octanyl)-*sn*-glycerophosphoethanolamine	1-Acyl,2-alkylglycerophosphoethanolamines
0.01041	1.89 higher in hypothermia	Unidentified	Unidentified
0.01041	1.59 higher in hypothermia	PE(16:0/22:6)	Diacylglycerophosphoethanolamines
0.01041	1.47 higher in hypothermia	Unidentified	Dialkylglycerophosphoglycerols
0.01041	1.75 higher in hypothermia	Unidentified	Unidentified
0.01041	1.92 higher in hypothermia	PI(16:0/18:0)	Diacylglycerophosphoinositols
0.01631	1.82 higher in hypothermia	PA(16:0/16:0)	Diacylglycerophosphates
0.01631	1.85 higher in hypothermia	Unidentified	Unidentified
0.01631	1.54 higher in hypothermia	PS(17:0/20:4)	Diacylglycerophosphoserines
0.01631	1.49 higher in hypothermia	Unidentified	Unidentified
0.01631	1.72 higher in hypothermia	Unidentified	Unidentified
0.01631	1.62 higher in hypothermia	Unidentified	Unidentified
0.02498	1.72 higher in hypothermia	PE(P-16:0/22:6)	1*Z*-alkenyl,2-acylglycerophosphoethanolamines
0.02498	1.54 higher in hypothermia	PS(18:0/18:1)	Diacylglycerophosphoserines
0.02498	1.47 higher in hypothermia	PS (20:0/18:2)	Diacylglycerophosphoserines
0.02498	1.82 higher in hypothermia	PG(18:0/20:4)	Diacylglycerophosphoglycerols
0.02498	1.82 higher in hypothermia	PG(18:0/22:6)	Diacylglycerophosphoglycerols
0.02498	1.52 higher in hypothermia	PI(16:0/18:1)	Diacylglycerophosphoinositols
0.02498	1.56 higher in hypothermia	PS (22:1/18:3)	Diacylglycerophosphoserines
0.03737	1.92 higher in hypothermia	Uunidentified	Unidentified
0.03737	1.61 higher in hypothermia	PS(16:0/18:1)	Diacylglycerophosphoserines
0.03737	1.92 higher in hypothermia	Unidentified	Unidentified
0.04461	1.89 higher in hypothermia	Unidentified	Unidentified

Table S1 Continued

(d) Class 3 (maintained at 27 °C for 6 h) compared with Class 5 (recovery at 37 °C for 2 h after a temperature of 27 °C for 6 h).

P value	Fold difference	Lipid identification	Lipid class
0.00649	4.23 higher in hypothermia	PS(18:0/18:1)	Diacylglycerophosphoserines
0.02498	2.48 higher in hypothermia	PA(16:0/18:1)	Diacylglycerophosphates
0.02498	3.23 higher in hypothermia	Unidentified	Diacylglycerophosphoinositols
0.02498	2.55 higher in hypothermia	Unidentified	Unidentified
0.02498	2.63 higher in hypothermia	Unidentified	Unidentified
0.02498	2.98 higher in hypothermia	Unidentified	Unidentified
0.02498	2.37 higher in hypothermia	Unidentified	Unidentified
0.03737	2.97 higher in hypothermia	PI(16:0/18:0)	Diacylglycerophosphoinositols
0.03737	2.25 higher in hypothermia	PS (22:1–18:3)	Diacylglycerophosphoserines
0.03737	2.24 higher in hypothermia	Unidentified	Unidentified
0.03737	2.33 higher in hypothermia	Unidentified	Unidentified
0.03737	2.75 higher in hypothermia	Unidentified	Unidentified

(e) Class 4 (maintained at 32 °C for 6 h) compared with Class 6 (recovery at 37 °C for 2 h after a temperature of 32 °C for 6 h).

P value	Fold difference	Lipid identification	Lipid class
0.003948	2.96 higher in hypothermia	PS(18:0/18:1)	Diacylglycerophosphoserines
0.006485	2.80 higher in hypothermia	Unidentified	Unidentified
0.006485	2.31 higher in hypothermia	PI(16:0/18:1)	Diacylglycerophosphoinositols
0.006485	2.52 higher in hypothermia	Unidentified	Unidentified
0.006485	2.16 higher in hypothermia	Unidentified	Unidentified
0.006485	2.4 higher in hypothermia	Unidentified	Unidentified
0.006485	2.5 higher in hypothermia	Unidentified	Unidentified
0.006485	2.12 higher in hypothermia	Unidentified	Unidentified
0.006485	2.39 higher in hypothermia	Unidentified	Unidentified
0.006485	2.46 higher in hypothermia	Unidentified	Unidentified
0.006485	2.43 higher in hypothermia	Unidentified	Unidentified
0.006485	2.33 higher in hypothermia	Unidentified	Unidentified
0.016309	1.91 higher in hypothermia	Unidentified	Unidentified
0.016309	1.9 higher in hypothermia	1,2-Dihexadecanoyl-*sn*-glycero-3-phosphosulfocholine	Glycerophospholipids
0.016309	1.80 higher in hypothermia	Unidentified	Dialkylglycerophosphoglycerols
0.016309	2.07 higher in hypothermia	PS(17:0/20:4)	Diacylglycerophosphoserines
0.016309	2.14 higher in hypothermia	PI(16:0/18:1)	Diacylglycerophosphoinositols
0.016309	2.02 higher in hypothermia	Unidentified	Unidentified
0.016309	2.81 higher in hypothermia	PI(16:0/18:0)	Diacylglycerophosphoinositols
0.016309	2.27 higher in hypothermia	PS (22:1/18:3)	Diacylglycerophosphoserines
0.016309	2.20 higher in hypothermia	Unidentified	Unidentified
0.016309	2.07 higher in hypothermia	Unidentified	Unidentified
0.016309	2.24 higher in hypothermia	PI(18:0/18:0)	Diacylglycerophosphoinositols
0.024975	1.96 higher in hypothermia	Unidentified	Unidentified
0.024975	2.00 higher in hypothermia	Unidentified	Unidentified
0.024975	1.91 higher in hypothermia	PS(18:0/18:1)	Diacylglycerophosphoserines
0.024975	1.70 higher in hypothermia	PE(18:0/22:6)	Diacylglycerophospho-ethanolamines
0.02846	2.11 higher in hypothermia	Unidentified	Unidentified
0.037373	1.68 higher in hypothermia	1-Tetrahexanoyl-2-(8-[3]-ladderane-octanyl)-*sn*-glycerophosphoethanolamine	1-Acyl,2-alkylglycerophosphoethanolamines
0.037373	1.76 higher in hypothermia	Unidentified	Unidentified
0.037373	1.77 higher in hypothermia	PE(16:0/22:6)	Diacylglycerophosphoethanolamines
0.037373	1.72 higher in hypothermia	Unidentified	Unidentified
0.037373	1.68 higher in hypothermia	Unidentified	Unidentified
0.0455	5.73 higher in hypothermia	Unidentified	Unidentified
0.0455	2.38 higher in hypothermia	PS(18:1/18:2)	Diacylglycerophosphoserines
0.0455	2.32 higher in hypothermia	Unidentified	Unidentified

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Low-Temperature Pausing of Cultivated Mammalian Cells

Lisa Hunt, David L. Hacker, Frédéric Grosjean, Maria De Jesus, Lorenz Uebersax, Martin Jordan, Florian M. Wurm

Swiss Federal Institute of Technology Lausanne, Faculty of Basic Sciences, Institute of Biological and Chemical Process Sciences, CH1015 Lausanne, Switzerland; telephone: 041.21.693.6141; fax: 041.21.693.6140; e-mail: Florian.Wurm@epfl.ch

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Abstract: There are currently two methods for maintaining cultured mammalian cells, continuous passage at 37°C and freezing in small batches. We investigated a third approach, the ''pausing'' of cells for days or weeks at temperatures below 37°C in a variety of cultivation vessels. High cell viability and exponential growth were observed after pausing a recombinant Chinese hamster ovary cell line (CHO-Clone 161) in a temperature range of 6–24°C in microcentrifuge tubes for up to 3 weeks. After pausing in T-flasks at 4°C for 9 days, adherent cultures of CHO-DG44 and human embryonic kidney (HEK293 EBNA) cells resumed exponential growth when incubated at 37°C. Adherent cultures of CHO-DG44 cells paused for 2 days at 4°C in T-flasks and suspension cultures of HEK293 EBNA cells paused for 3 days at either 4°C or 24°C in spinner flasks were efficiently transfected by the calcium phosphate-DNA coprecipitation method, yielding reporter protein levels comparable to those from nonpaused cultures. Finally, cultures of a recombinant CHO cell line (CHO-YIgG3) paused for 3 days at 4°C, 12°C, or 24°C in bioreactors achieved the same cell mass and recombinant protein productivity levels as nonpaused cultures. The success of this approach to cell storage with rodent and human cell lines points to a general biological phenomenon which may have a wide range of applications for cultivated mammalian cells. © 2004 Wiley Periodicals, Inc.

Keywords: CHO-DG44 cells; HEK293E cells; green fluorescent protein; IgG antibody; hypothermia; cell storage

INTRODUCTION

Two ways are known to provide cultivated mammalian cells for experimental work. Cells are either continuously propagated under stringent control of temperature and other environmental conditions, requiring constant attention, or limited amounts of cells are stored frozen for extended times (Mazur, 1970). Reestablishing a culture from a stock of frozen cells requires days if not weeks to reach the quantity of cells needed for most applications. Considering these two options, the versatility of mammalian cell culture would be improved if cells could be routinely kept at low temperatures with little or no maintenance and then recovered quickly without a decline in their capacity for growth. Here it is demonstrated that adherent or suspension cultures of mammalian cells can be paused for short periods (days to weeks) in standard media at temperatures between 4°C and 24°C.

The mammalian constraint in body temperature around 37°C has most likely resulted in a large number of diverse molecular adaptations affecting many aspects of cellular activity. However, cells, organs, and even entire organisms can recover from heat and cold stress, seemingly without any long-term consequences. In fact, periodic hypothermic exposure of cultivated mammalian cells can result in cold-tolerant cell lines (Michl et al., 1966; Glofcheski et al., 1993). Mammalian cells are therefore likely to possess a set of natural response mechanisms when diverting from and returning to the 37°C setpoint. Tissues such as skin and testis are normally maintained at temperatures lower than that of the body cavity, and the core temperature of long-term hibernators is allowed to vary during periods of hibernation (Willis, 1987). Hypothermia also has several medical applications. For example, transplantable tissues are often preserved at low temperatures for short periods (Sicular and Moore, 1961; Belzer et al., 1967). Reduction of the heart's temperature during cardiac surgery is known to reduce the risk of myocardial ischemia (Mauny and Kron, 1995), and mild hypothermia is used to limit the severity of traumatic brain injuries (Connolly et al., 1962; Marion et al., 1997).

For cultured mammalian cells, cold exposure reduces the rate of ATP synthesis and alters membrane permeability (Hochachka, 1986; Willis, 1987). Mild hypothermia (25–33°C) reduces the rate of progression through the cell cycle, while moderate (16–20°C) or severe hypothermia (4–10°C) may block the cell cycle in the G_2 phase or at the G_1/S boundary, respectively (Rieder and Cole, 2002). Transcription and translation are reduced by cold exposure, but the expression of a few proteins including p53, WAF1,

Correspondence to: Florian M. Wurm

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and cold-inducible RNA-binding protein (CIRP) is elevated at temperatures in the range of 25–33°C (Nishiyama et al., 1997; Matijasevic et al., 1998; Ohnishi et al., 1998; Sonna et al., 2002). Interestingly, heat shock proteins are expressed during rewarming to 37°C after hypothermic exposure (Holland et al., 1993; Liu et al., 1994; Kaneko et al., 1997). Cold stress also results in protein denaturation and aggregation and disruption of the cellular cytoskeleton (Fujita, 1999; Sonna et al., 2002). Cold stress can induce apoptosis, but this may be cell-type-specific and dependent on the length and severity of the hypothermic exposure (Soloff et al., 1987; Perotti et al., 1990; Kruman et al., 1992; Gregory and Milner, 1994; Grand et al., 1995; Rauen et al., 2000).

As with other forms of cell stress, mammalian cells can recover from hypothermic exposure. We took advantage of this property to demonstrate that cultivated mammalian cells can be stored for up to 3 weeks at 4–24°C and then recovered by rewarming to 37°C. We use the term pausing to describe this method of cell storage. To be useful, pausing should be possible at any phase of a cell culture and at any scale of operation. Both adherent and suspension cells were paused in commonly used culture vessels such as T-flasks, spinner flasks, and bioreactors. After pausing, viable cells continued to divide and were used for routine applications, including stable and transient recombinant gene expression. These findings suggest that pausing is an attractive alternative for the short-term storage of cultivated mammalian cells.

MATERIALS AND METHODS

Cells

Adherent CHO-DG44 and HEK293 EBNA (HEK293E) cells were maintained in DMEM/F12 medium with 2% fetal calf serum (FCS). For CHO-DG44 cells the medium also contained 0.68 g/l hypoxanthine and 0.194 g/l thymidine (Sigma Chemical, St. Louis, MO). Suspension-adapted HEK293E cells were grown in spinner flasks in serum-free Ex-Cell 293 medium (JRH Biosciences, Lenexa, KS) supplemented with 4 mM glutamine. The agitation speed was 90 rpm. Recombinant CHO-Clone 161 cells expressing the enhanced green fluorescent protein (GFP) were grown as an adherent culture in DMEM/F12 medium in the presence of 2% FCS (Hunt et al., 1999). Suspension cultures of recombinant CHO-YIgG3 cells that express the enhanced yellow fluorescent protein (YFP) and a human IgG were maintained in spinner flasks stirred at 90 rpm in serum-free ProCHO5-CDM medium (Cambrex BioScience, Walkersville, MD) supplemented with 4 mM glutamine (Miescher et al., 2000; Hunt et al., 2002).

Pausing in Microcentrifuge Tubes

CHO-Clone 161 cells at mid-log phase were trypsinized, harvested by centrifugation, washed in phosphate-buffered saline (PBS), resuspended at a density of 1×10^6 cells/ml in fresh DMEM/F12 medium supplemented with 2% FCS and 4 mM glutamine, and transferred as 1-ml aliquots into 1.5 ml microcentrifuge tubes. The cells were incubated in the absence of CO_2 control for various times at the temperatures indicated in the text. Subsequently, 30 or 50 µl aliquots of the paused cells were transferred to 12-well microtiter plates supplied with 0.5 ml of fresh DMEM/F12 medium containing 2% FCS and 4 mM glutamine per well. For assessing cell growth, the samples were incubated at 37°C, and fluorescence was measured at various times with a Cytofluor Series 4000 plate-reading fluorometer (PerSeptive Biosystems; Framingham, MA) using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Cell viability was determined by the Trypan blue exclusion method.

Pausing in T-flasks

Adherent CHO-DG44 and HEK293E cells were seeded in 25 cm^2 T-flasks in DMEM/F12 medium supplemented with 2% FCS and incubated at 37°C in 5% CO_2 and 95% humidity. When the cultures reached 80% confluence the caps of the flasks were closed and the cultures were stored at 4°C for 9 days. After pausing, the cells were incubated for 12 h at 37°C under humidity and CO_2 control. Adherent cells were detached with trypsin and 2×10^5 cells were seeded in 25 cm^2 T-flasks in 5 ml of fresh medium with 2% FCS and incubated at 37°C under humidity and CO_2 control. At various times the cells were trypsinized and counted using a CASY1 counter (Schärfe System, Reutlinger, Germany).

DNA Transfections

Adherent CHO-DG44 cells were grown in T-150 flasks to 80% confluence at 37°C in 5% CO_2 and 95% humidity. The cap of the flask was then closed and the cells were stored at 4°C for 2 days. After pausing, the cultures were incubated at 37°C for 5 h. The cells were trypsinized and seeded in 12-well microtiter plates at a density of 4×10^5 cells/ml in 1 ml of modified DMEM/F12 medium with 2% FCS, 4 mM glutamine, 0.68 g/l hypoxanthine, and 0.194 g/l thymidine. Nonpaused control cultures maintained in T-flasks at 37°C were seeded under the same conditions. After 4 h of incubation at 37°C, 660 µl of the medium was removed and 100 µl of a calcium phosphate-DNA coprecipitate containing 2.5 µg of pCMV-DsRed-Express (ClonTech, Palo Alto, CA) was added to each well. After 1 h of incubation at 37°C, the medium was removed and the cells where exposed to an osmotic shock by the addition of 10% glycerol in PBS. After 1 min, the glycerol solution was removed and replaced with fresh medium. After 3 days of incubation at 37°C DsRed expression was determined with a plate-reading fluorometer.

Suspension cultures of HEK293E cells were seeded in spinner flasks at a density of 5×10^5 cells/ml in 300 ml of

Ex-Cell 293 medium. After incubation at 37°C for 1 day, the cultures were incubated at 4°C or 24°C for 3 days. A control culture was maintained at 37°C. Paused and nonpaused cells were then passed to fresh Ex-Cell 293 medium in spinner flasks at a density of 1×10^6 cells/ml and incubated at 37°C for 24 h. Cells were then seeded in 12-well microtiter plates at a density of 5×10^5 cells/ml in 1 ml of modified DMEM/F12 medium with 1% FCS. To each well, 100 µl of calcium phosphate-DNA coprecipitate containing 50 ng pEGFP-N1 (ClonTech) and 2.45 µg calf thymus DNA (Invitrogen, Basel, Switzerland) was added. The plates were agitated at 120 rpm for 4 h at 37°C in 5% CO_2 and 95% humidity. One volume of Pro293s-CDM medium (Cambrex BioScience) was then added to each well. At 3 days posttransfection the cells were lysed by addition of 200 µl PBS with 10% Triton X-100. After a 1-h incubation with agitation at 37°C, the GFP level was determined using a plate-reading fluorometer as described above.

Pausing in Bioreactors

Each 3-L bioreactor (Applikon, The Netherlands) was seeded with CHO-YIgG3 cells at a density of 5×10^5 cells/ml in 1 l of ProCHO5 CDM medium. The cultures were maintained at 37°C at pH 7.1 with the dissolved O_2 maintained at 20%. Agitation was set at 150 rpm. For paused cultures, the temperature was reduced at 6 h postinoculation for a period of 72 h. Pausing at 24°C was accomplished by disconnection of the heating unit. The room temperature during any single experiment fluctuated by ±1°C. Cultures were maintained at either 4°C or 12°C using a 3-L water-jacketed bioreactor (Applikon) connected to a MultiTemp III waterbath (Amersham Biosciences, Uppsala, Sweden). During pausing, the pH was maintained at 7.1. To reduce the risk of cell damage during pausing, the stirring speed was reduced to 90 rpm, the lowest speed that prevented settling of the cells. After pausing the temperature was returned to 37°C and stirring was increased to 150 rpm. Samples were taken once or twice per day. The cell number and viability were determined using the Trypan blue exclusion method. The packed cell volume (PCV) was determined using 1 ml PCV tubes (Techno Plastic Products, Trasadingen, Switzerland). The concentration of fully assembled IgG in the culture medium was determined by sandwich ELISA as previously described (Meissner et al., 2001). Glucose, glutamine, sodium bicarbonate, and sodium hydroxide were added to the cultures as needed.

RESULTS

Pausing in Microcentrifuge Tubes

Our initial studies to investigate the feasibility of pausing utilized recombinant CHO-Clone 161 cells that homogeneously express GFP. For this cell line, a linear correlation between cell number and fluorescence has been observed (Hunt et al., 1999). Therefore, it was possible to use a standard multiwell plate reader to noninvasively monitor growth of these cultures over time. Adherent CHO-Clone 161 cells were trypsinized, suspended in fresh medium, transferred to microcentrifuge tubes, and stored at various temperatures for 4 days without agitation. After pausing, the highest viability was observed in cultures that were maintained at 17°C, but high viability was also seen in cultures stored at 6°C and 22°C (Fig. 1A). In contrast, pausing at 0°C or 37°C resulted in a high percentage of nonviable cells (Fig. 1A). Paused cells were evaluated for growth at 37°C by dilution of 30 µl aliquots of paused cultures into fresh medium in 12-well microtiter plates. The extent of cell growth at 37°C was determined by monitoring the level of GFP expression. Vigorous growth was observed following pausing at 6–22°C, while cells

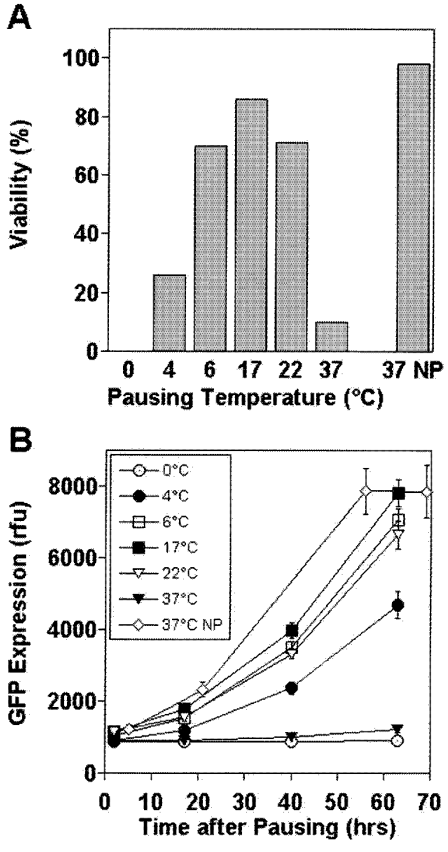

Figure 1. Pausing of CHO-Clone 161 cells in microcentrifuge tubes. **A:** Adherent cells were trypsinized and paused as suspension cultures in microcentrifuge tubes for four days at various temperatures as indicated. The cultures were not agitated during pausing. The nonpaused (NP) culture was maintained in a T-flask at 37°C. The viability of the cultures was determined by the Trypan blue exclusion method. **B:** Cells from paused and nonpaused (NP) cultures were passed in duplicate to fresh medium in 96-well microtiter plates and maintained at 37°C. GFP expression was measured by fluorometry at the times indicated.

paused at 4°C grew more slowly (Fig. 1B). Little growth was observed for cells paused at 0°C or 37°C (Fig. 1B).

The effect of the length of the pausing period on cell viability was investigated by maintaining CHO-Clone 161 cells at 6°C for up to 20 days. The viability of the paused cultures decreased with time, but more than 30% of the cells remained viable after 20 days of storage (Fig. 2A). After pausing, 50 μl aliquots of cultures were diluted in fresh medium in 12-well microtiter plates and incubated at 37°C. All of the cultures resumed growth, albeit with a lag period that was most pronounced for cells stored for 6 or 20 days (Fig. 2B). After the lag period, the paused cells grew at approximately the same rate as the nonpaused cells (Fig. 2B). Although the cultures paused for 6 or more days did not reach the same cell density as the nonpaused control, cells paused for 2 days at 6°C grew at approximately the same rate and to the same cell density as the nonpaused culture (Fig. 2B).

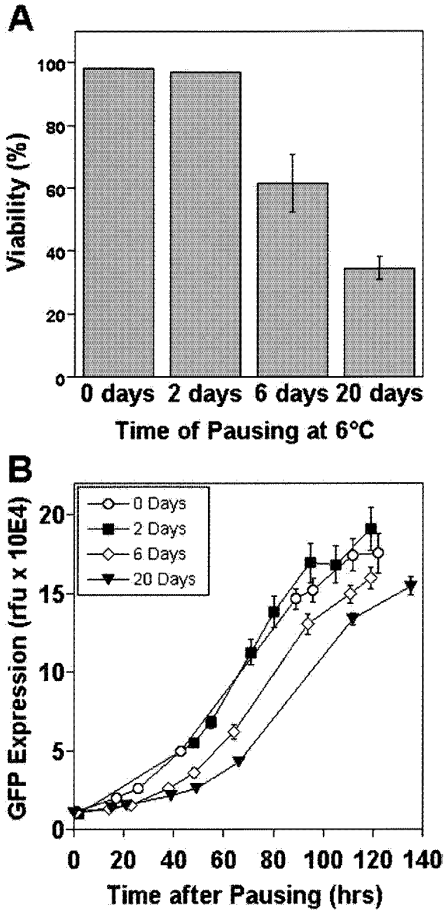

Figure 2. Pausing of CHO-Clone 161 cells in microcentrifuge tubes. **A:** Cells were paused as described in Figure 1 at 6°C for various times as indicated. The nonpaused (0 days) cells were maintained in a T-flask at 37°C. The viability of each culture was determined by the Trypan blue exclusion method. Each bar represents the average of two independent cultures. **B:** Paused and nonpaused (NP) cultures were passed at various times to 96-well microtiter plates and incubated at 37°C. The GFP expression was measured by fluorometry at the times indicated. Each point represents the average of three cultures.

Pausing in T-flasks

To determine if the results observed with CHO-Clone 161 cells applied to other cells, adherent HEK293E and CHO-DG44 cells were paused in T-flasks at 4°C for 9 days. During pausing, the cells became round and detached from the surface even though serum was present in the medium. This may have been due to the disassembly of the cytoskeleton (Sonna et al., 2002). After pausing, the cultures were incubated at 37°C overnight. During this period the viable cells reattached to the plate and regained their fibroblastic appearance. Adherent cells were then trypsinized, replated in fresh medium, and incubated at 37°C. For the paused cells, exponential growth did not begin until about 24 h after plating (Fig. 3). In contrast, the cell number in the nonpaused cultures more than doubled during this period (Fig. 3). By 93 h after plating, the paused cultures had reached about the same cell density as the control cultures (Fig. 3). Similar results were observed with adherent cultures of baby hamster kidney (BHK) cells (data not shown). These results demonstrate that commonly used human and rodent cell lines can be stored at reduced temperatures for several days.

Transfection of Paused Cells

To demonstrate the utility of pausing, we determined if paused cells could be transfected at the same efficiency as nonpaused control cells. Adherent CHO-DG44 cells were grown in T-flasks to 80% confluence at 37°C and then incubated at 4°C for 2 days. After pausing, the cells

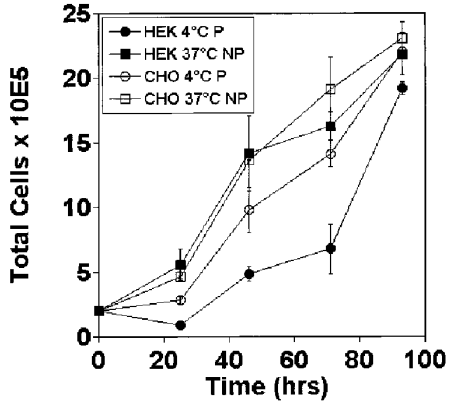

Figure 3. Pausing of adherent cells in T-flasks. Duplicate cultures of HEK293E and CHO-DG44 cells were paused in 25 cm^2 T-flasks at 4°C for 9 days. The viable cells were allowed to reattach by incubation at 37°C for 12 h. Paused (P) and nonpaused (NP) cells were then trypsinized, plated T-flasks, and incubated at 37°C. The cell number was determined with a CASY1 counter at the times indicated.

were incubated at 37°C for 5 h to allow viable cells to reattach to the plate. The viability of the paused culture was 93%. The cells were passed to 12-well microtiter plates and transfected with pCMV-DsRed-Express using the calcium phosphate-DNA coprecipitation method. As shown in Figure 4A, the level of DsRed expression at 3 days posttransfection was slightly higher in the paused culture than in the nonpaused culture (Fig. 4A). These results demonstrated that pausing for 2 days at 4°C did not alter the transfection efficiency of adherent CHO-DG44 cells.

To determine if this observation applied to other cell lines, a similar experiment was performed with suspension-adapted HEK293E cells in spinner flasks. The cultures were incubated at either 4°C or 24°C for 3 days. After pausing, the cells were passed to fresh medium at a density of 1×10^6 cells/ml and maintained at 37°C for 1 day. The cells were passed to 12-well microtiter plates and transfected with pEGFP-N1 using the calcium phosphate-DNA coprecipitation method. At 3 days posttransfection, GFP expression in cells paused at either 4°C or 24°C was similar to the level observed in nonpaused cells (Fig. 4B). Similar results were obtained when the cells were paused for 4 days at either 4°C or 24°C (data not shown). Through 4 days of pausing at either temperature, the viability of the cultures in spinner flasks remained above 85%. After 4 days of pausing, however, the viability decreased to about 75% and continued to decrease with further exposure to low temperature. These results demonstrate that suspension-adapted HEK293E cells paused in spinner flasks retained their capacity for transfection for up to 4 days.

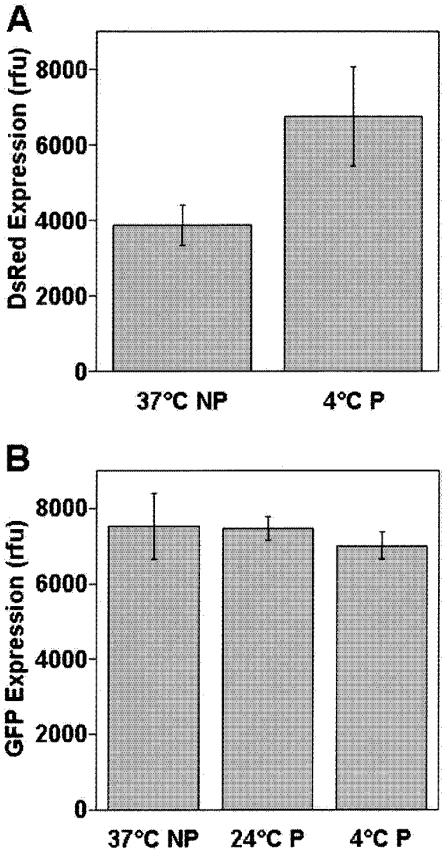

Figure 4. Transfection of paused cells. **A:** Adherent CHO-DG44 cells were paused (P) for 2 days at 4°C or maintained at 37°C (NP), passed to 12-well microtiter plates, and transfected with pCMV-DsRed-Express. DsRed expression was measured at 3 days post-transfection. Each bar represents the average of 11 transfections. **B:** Suspension cultures of HEK293E cells were paused (P) in spinner flasks for 3 days at the temperatures indicated. The nonpaused (NP) cultures were maintained at 37°C. After pausing, the cells were transfected with pEGFP-N1 in microtiter plates. GFP expression was measured at 3 days posttransfection. Each bar represents the average of three transfections.

Pausing in Bioreactors

We also explored the possibility of pausing cells in a bioreactor using a recombinant CHO cell line (CHO-YIgG3) that expresses both YFP and a human IgG (Miescher et al., 2000; Hunt et al., 2002). A single homogenous seed culture was used to inoculate three 3-L bioreactors at a density of 0.5×10^6 cells/ml. One of the reactors was maintained at 37°C and the other two were paused at either 12°C or 24°C beginning at 6 h postinoculation. After 72 h at the low temperature (78 h postinoculation), the temperature was returned to 37°C. The paused cells grew exponentially following a short lag period, but they did

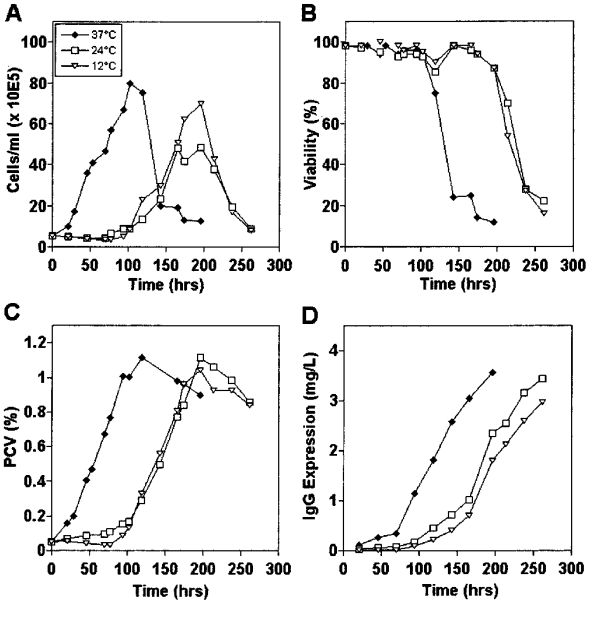

Figure 5. Pausing of CHO-YIgG3 cells in bioreactors. Suspension cultures of CHO-YIgG3 cells in 3-L bioreactors were paused at 12°C or 24°C. Pausing began at 6 h postinoculation and ended at 78 h postinoculation. The control culture was maintained at 37°C throughout the experiment. The viable cell number (**A**), viability (**B**), PCV (**C**), and IgG titer (**D**) were measured at the times indicated.

not achieve the same maximum cell density as the control culture (Fig. 5A). During pausing and after rewarming to 37°C, cell viability did not decrease (Fig. 5B). The onset of the viability decline of the paused cultures occurred ~3 days after that of the control culture (Fig. 5B). The PCV of the two paused cultures reached the same level as that of the control, indicating that the three cultures attained the same cell mass (Fig. 5C). These results suggest that the average cell size in the paused cultures was greater than that in the nonpaused cultures. Finally, the IgG titer in the culture paused at 24°C reached about the same level as in the control culture, while that in the culture paused at 12°C was slightly lower than the control (Fig. 5D).

In a separate experiment CHO-YIgG3 cells were paused in a 3-L bioreactor at 4°C for 72 h. After the temperature was raised to 37°C the paused culture eventually achieved the same maximum cell density as the control (Fig. 6A). The viability of the paused culture was stable during the period of pausing, but a 70% loss in viability was observed after rewarming to 37°C at 78 h postinoculation (Fig. 6B). However, the viability of the culture eventually returned to 90% (Fig. 6B). The PCV of the paused and nonpaused cultures also reached the same level (Fig. 6C). The IgG titer was slightly higher for the paused culture than for the nonpaused culture (Fig. 6D). These results demonstrate that a recombinant CHO cell line can be paused at 4–24°C for up to 3 days without substantial negative effects on cell mass or recombinant protein expression.

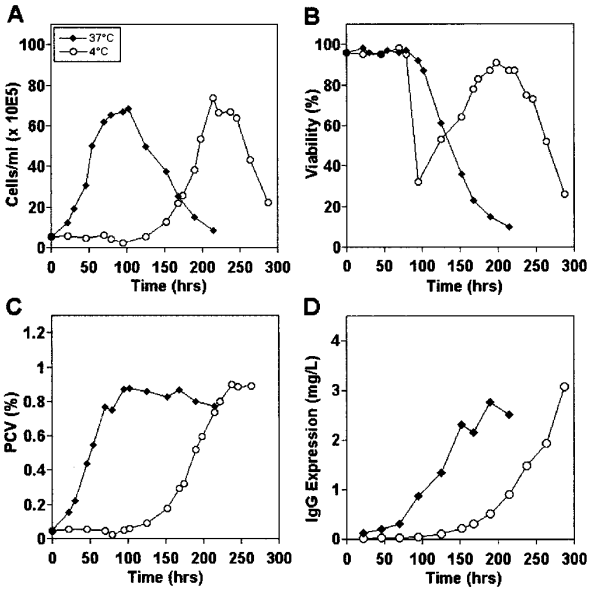

Figure 6. Pausing of CHO-YIgG3 cells in bioreactors. A suspension culture of CHO-YIgG3 cells was paused at 4°C in a 3-L bioreactor. Pausing began at 6 h postinoculation and ended at 78 h postinoculation. The control culture was maintained at 37°C throughout the experiment. The viable cell number (**A**), viability (**B**), PCV (**C**), and IgG titer (**D**) were measured at the times indicated.

DISCUSSION

The experiments described here demonstrate that cultured mammalian cells stored for short times (days or weeks) at low temperatures (4–24°C) resumed exponential growth when rewarmed to 37°C, albeit with a lag period whose duration varied depending on the pausing conditions and the cell line. This represents a new approach to the short-term storage of cultivated mammalian cells. Cells were paused for up to 3 weeks in this range of temperatures, and pausing was performed in a number of different culture vessels including T-flasks, spinner flasks, and bioreactors. For the two applications tested, transient gene expression following transfection and recombinant protein expression from a stable cell line, the paused cells performed as well as the nonpaused control cells in most cases.

As one example of the utility of this method of storage, suspension cultures of CHO-YIgG3 cells were paused for 3 days in bioreactors. During pausing at 4°C, 12°C, or 24°C the cell viability ranged from 93–98%. Rewarming to 37°C was only detrimental to cells paused at 4°C. In this case, the viability was reduced to 30% by 16 h after the return to 37°C. Cell death in this instance may have been due to apoptosis, but this was not confirmed. The paused cells were visibly smaller immediately after rewarming than before, one of the characteristics of apoptotic cells (Hockenberry, 1995). Cold-induced apoptosis has been shown to result from increases in the intracellular pools of chelatable iron and reactive oxygen species (Rauen et al., 1999, 2000). Thus, it may be possible to prevent cell damage due to exposure to 4°C by including antioxidants in the culture medium (Rauen et al., 2000). Clearly, cold-induced apoptosis was not a significant problem with the cultures paused in bioreactors for 3 days at 12°C or 24°C. This is not surprising, since two distinct mechanisms of hypothermic damage have been reported for cells exposed to temperatures above or below the minimum inactivation temperature, which is usually between 5–10°C for cultivated mammalian cells (Kruuv et al., 1995). Below this temperature, direct chilling injury (DCI) caused by hypothermic exposure is linked to thermotropic phase transitions of lipids resulting in loss of membrane integrity (Arav et al., 1996).

Despite the differences in cell viability after rewarming, the cultures paused in bioreactors produced approximately the same level of recombinant antibody as the nonpaused control cultures. These results suggest that stable cell lines can be paused at low temperatures for short periods without compromising their ability to produce recombinant protein. We have also shown that adherent CHO-DG44 cells paused and suspension-adapted HEK293E cells retain the capacity to be efficiently transfected using the calcium phosphate-DNA coprecipitation method. For CHO-DG44 cells, an elevation of reporter gene expression was consistently observed for the paused cells as compared to the nonpaused cells. This may have been a consequence of cell synchronization caused by pausing at 4°C (Rieder

and Cole, 2002). Efficient transfection of adherent CHO-DG44 by this method has been shown to be cell cycle-dependent (Grosjean et al., 2002).

Although the feasibility of pausing has been demonstrated, we have not yet attempted to optimize the method. Preliminary experiments have suggested that the pH and the availability of glucose and glutamine are important parameters for long-term pausing, but pausing for 1–2 days can be performed in PBS (Hunt and Wurm, unpubl. data). Additional experimentation will be necessary to determine how to best maintain cell viability after pausing. Our initial studies, however, do suggest that there are limits to this approach to cell storage. For example, rewarming of cells stored at 4°C had a significant negative effect on cell viability. Despite limitations, low temperature pausing is expected to be beneficial to many users of mammalian cell culture, as it provides a simple and inexpensive method for the short-term storage of cells in a number of different formats. It should be possible to transport cells for several days without temperature control. In addition, the observation that adherent cells detach during pausing suggests that this approach can be used instead of enzymatic or mechanical detachment if these techniques are not feasible.

This article is dedicated to the memory of Dr. Lisa Hunt, who died on September 21, 2002. The authors thank Ilda Tabuas Baieta Muller, Patrizia Tromba, and Hicham El Abridi for technical assistance, Martin Bertschinger for preparation of the figures, and Techno Plastic Products for the gift of the PCV tubes.

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Effect of Low Culture Temperature on Specific Productivity and Transcription Level of Anti-4-1BB Antibody in Recombinant Chinese Hamster Ovary Cells

Sung Kwan Yoon, Sung Hyun Kim, and Gyun Min Lee*

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 371-1 Kusong-Dong, Yusong-Gu, Daejon 305-701, Korea

Lowering the culture temperature has been suggested as a useful tool for improving the production of recombinant proteins in Chinese hamster ovary (CHO) cells. In an effort to improve anti-4-1BB antibody production in recombinant CHO (rCHO) cells, rCHO cells producing anti-4-1BB antibody (LGA31-56) were cultivated at three different temperatures, 30, 33, and 37 °C. Lowering the culture temperature led to suppressed cell growth, cell cycle arrest in G_0/G_1 phase, and improved cell viability for a longer period. However, antibody production and q_{Ab} were not increased at low culture temperature. The maximum antibody concentration and q_{Ab} at 37 °C were 110.6 ± 2.6 μg mL^{-1} and 0.43 ± 0.03 μg (10^6 cells h)$^{-1}$, respectively, whereas those at 30 °C were 28.3 ± 3.8 μg mL^{-1} and 0.44 ± 0.07 (10^6 cells h)$^{-1}$, respectively. Northern blot analysis revealed that lowering the culture temperature did not increase the transcription level of heavy and light chains. These results were quite in contrast with the improved production of erythropoietin, which is expressed in the same CHO host and driven by the same CMV promoters, by lowering the temperature. Taken together, the results obtained imply that the beneficial effect of low culture temperature on recombinant protein production in rCHO cells is cell-line-specific.

Introduction

Chinese hamster ovary (CHO) cells are being used increasingly in industry to manufacture therapeutic antibodies (*1*). To realize an efficient process for antibody production by CHO cell culture, environmental parameters that affect cell growth and antibody production need to be investigated.

To simulate normal body temperature, recombinant CHO (rCHO) cells are cultivated at 37 °C. Lowering the culture temperature below 37 °C has been recognized as a means to improve culture performance (*2*–*7*). rCHO cell culture at low temperature decreases specific growth rate (μ) but improves cell viability and decreases the rate of releasing cellular proteins for a longer period (*2*). Moreover, lowering the culture temperature may also increase specific productivity (q), though its effect on q is variable among different rCHO cell lines. For example, cultivation of rCHO cells at low temperature resulted in enhanced q of C-terminal α-amidating enzyme (*3*), tissular plasminogen activator (t-PA) (*4*), secreted alkaline phosphatase (SEAP) (*5*), and erythropoietin (EPO) (*6*) but did not affect q of TNK-tPA and decreased q of rhesus thrombopoietin (TPO) (*8*).

Anti-4-1BB monoclonal antibody, a humanized antibody produced from CHO cells, is a potential therapeutic antibody controlling unwanted immune responses in persons with autoimmune diseases (*9*). To improve anti-4-1BB antibody production from rCHO cells (LGA31-56), we investigated the effect of culture temperature (30, 33, and 37 °C) on cell growth and antibody production. Furthermore, to understand the effect of low culture temperature on specific antibody productivity (q_{Ab}), we analyzed the transcription level of immunoglobulin (Ig) and cell cycle of rCHO cells.

* To whom correspondence should be addressed. Fax: 82-42-869-2610. E-mail: gmlee@mail.kaist.ac.kr.

Materials and Methods

Cell Line and Cell Culture. The rCHO cells producing a humanized anti-4-1BB antibody for active suppression of antibody-mediated autoimmune reactions (LGA31-56) were used in this study. Heavy-chain (HC) and light-chain (LC) expression vectors were constructed separately, as described previously (*9*). Then they were cotransfected into dihydrofolate reductase (DHFR)-deficient CHO cells (DUKX-B11, ATCC CRL-9096) and underwent subsequent DHFR/methotrexate (MTX)-mediated gene amplification. The stable rCHO cells (LGA31-56) were selected at 1 μM MTX.

The medium for culture maintenance was Iscove's modified Dulbecco's medium (IMDM, Gibco, Grand Island, NY) supplemented with 10% (v/v) dialyzed fetal bovine serum (dFBS, Gibco). Cells were maintained as monolayer cultures in 25 cm^2 T-flasks (Nunc, Roskilde, Denmark) in a humidified 5% CO_2 incubator at 37 °C. Exponentially growing cells were inoculated at 0.6×10^5 cells mL^{-1} into 25 cm^2 T-flasks containing 4 mL of IMDM supplemented with 10% dFBS, and the T-flasks were incubated in the humidified 5% CO_2 incubator at 37 °C. When the viable cell concentration reached approximately 1×10^5 cells mL^{-1}, the medium was removed and the cells were washed once with Dulbecco's phosphate-buffered saline (D-PBS, Gibco). The T-flasks were then filled with 4 mL of SF2 serum-free medium (*10*) and were incubated in humidified 5% CO_2 incubators at three

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different temperatures, 30, 33, and 37 °C, respectively. Periodically, T-flasks were sacrificed to determine viable cell concentration. Culture supernatants, after centrifugation, were aliquoted and kept frozen at −70 °C for later analyses. The viable cell concentration was estimated by using the trypan blue dye exclusion method.

Quantitation of Anti-4-1BB Antibody. The secreted anti-4-1BB antibody concentration was quantified by an enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates (Nunc) were coated with bacterially expressed 4-1BB, which is fused to glutathione *S*-transferase (GST-4-1BB) and blocked with bovine serum albumin (BSA) and Tween 20. The human IgG standard (Sigma, St. Louis, MO) and culture supernatants diluted with blocking buffer were loaded on wells and treated with goat anti-human IgG peroxidase conjugate (Sigma) in diluent solution.

Cell Cycle Analysis. To assess the effect of culture temperature on cell cycle distribution, cellular DNA contents were analyzed by flow cytometry, as described previously (*6*).

Northern Blot Analysis. For Northern analysis, total RNA was extracted from the cells using TRI reagent (Sigma) according to the manufacturer's protocol. After total RNA (1.5 μg) was developed on 1.2% agarose formaldehyde gels by electrophoresis, HC and LC mRNAs were characterized by Northern blot hybridization with the respective probes. The HC and LC probes were radioactively labeled by random primed incorporation of [α-^{32}P]dCTP (Amersham, Amersham, U.K.). Membrane transfer, prehybridization, and hybridization were performed using the protocol described previously (*11*). After hybridization, the band intensity was quantitated using PhosphorImager (Molecular Dynamics). Subsequently, the membrane was stripped and rehybridized with a CHO β-actin cDNA probe for band intensity normalization. Hybridization and analysis with the β-actin probe were performed in the same manner.

Evaluation of Specific Antibody Productivity. The q_{Ab} was based on the data collected until the end of stationary phase and was evaluated from a plot of the anti-4-1BB antibody concentration against the time integral values of the growth curve (*12*).

Results and Discussion

To determine the effect of low culture temperature on rCHO cells (LGA31-56) in regard to growth and anti-4-1BB antibody production, cells were cultivated at three different temperatures, 30, 33, and 37 °C. Duplicate cultures were performed two separate times.

Figure 1 shows typical cell growth and viability profiles during culture. When cells were subjected to low temperature, cell growth was suppressed. The maximum viable cell concentration and μ at 37 °C were 2.53 ± 0.20 × 10^6 (av ± SD, n = 2) cells mL^{-1} and 0.022 ± 0.003 h^{-1}, respectively. On the other hand, those at 33 °C were 1.31 ± 0.11 × 10^6 cells mL^{-1} and 0.014 ± 0.004 h^{-1}, respectively. At 30 °C, the maximum viable cell concentration obtained was only 0.31 ± 0.06 × 10^6 cells mL^{-1}.

To further investigate the growth suppression at low culture temperature, cell cycle distribution was analyzed by flow cytometry. As observed in other rCHO cells (*5*, *6*), lowering the culture temperature resulted in rapid reduction of cells in S phase and concomitant accumulation of cells in G_0/G_1 phase. After 78 h cultivation, approximately 77% of cells and 67% of cells accumulated in G_0/G_1 phase at 30 and 33 °C, respectively, while 45% of cells remained in G_0/G_1 phase at 37 °C (Table 1). These

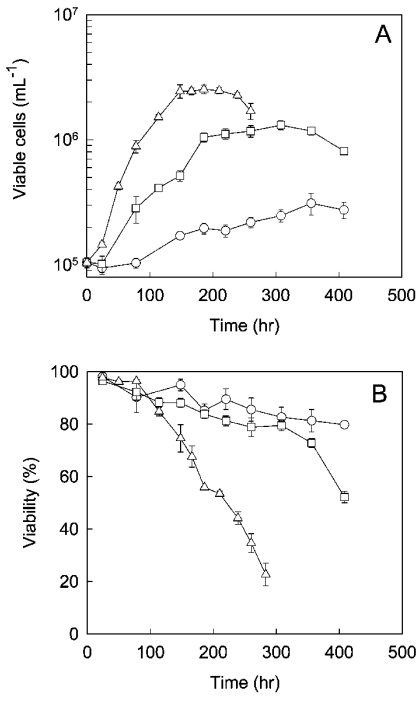

Figure 1. Effect of culture temperature on cell growth and viability: (○) 30 °C; (□) 33 °C; (△) 37 °C. (A) Viable cell concentration. (B) Cell viability. The error bar represents the standard deviations calculated from the data (n = 4).

Table 1. Effect of Culture Temperature on Percent of Cells in Different Phases of the Cell Cycle of rCHO Cells[a]

	culture temperature (°C)								
	30			33			37		
time (h)	G_0/G_1 (%)	S (%)	G_2/M (%)	G_0/G_1 (%)	S (%)	G_2/M (%)	G_0/G_1 (%)	S (%)	G_2/M (%)
24	64.9	20.2	14.9	59.1	28.3	12.6	36.0	52.6	11.4
78	77.4	17.4	5.2	66.6	15.6	17.8	44.8	45.1	10.0

[a] Cell samples were taken during cultures shown in Figure 1.

results agreed that low culture temperature led to suppressed cell growth, cell cycle arrest in G_0/G_1 phase, and improved cell viability for a longer period in batch culture (*2*–*7*).

A cold-inducible RNA-binding protein (CIRP) may play an essential role in suppression of CHO cell growth at low culture temperature. In response to low culture temperature (32 °C), expression of CIRP in mouse fibroblasts was induced, and growth was suppressed. By suppressing the induction of CIRP with antisense oligodeoxynucleotides, this growth suppression was alleviated, while overexpression of CRIP resulted in suppressed cell growth at 37 °C with prolongation of G_0/G_1 phase (*13*). However, the molecular mechanism underlying the cold response in CHO cells needs to be elucidated.

Figure 2A shows antibody concentration in the medium during the cultures shown in Figure 1A. Lowering the culture temperature did not increase antibody production. The maximum antibody concentration at 37, 33, and 30 °C was 110.6 ± 2.6, 106.4 ± 8.6 and 28.3 ± 3.8 μg mL^{-1}, respectively. To determine q_{Ab}, the time integral of viable cells versus antibody concentration was plotted as shown in Figure 2B. The slope of such plots is equal to q_{Ab}, if q_{Ab} is constant. The q_{Ab} was fairly constant until the end of the stationary phase, though the q_{Ab} at 33 and

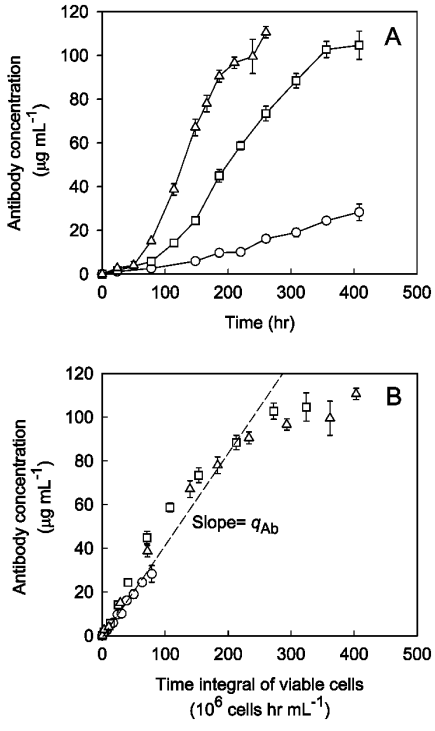

Figure 2. Effect of culture temperature on anti-4-1BB antibody production and q_{Ab}: (○) 30 °C; (□) 33 °C; (△) 37 °C. (A) Antibody concentration. (B) Evaluation of q_{Ab}. The q_{Ab} is equal to the slope of the plot. The error bar represents the standard deviations calculated from the data ($n = 4$).

37 °C decreased during the decline phase of growth. Therefore, the q_{Ab} was calculated until the end of the stationary phase (Table 2). Like the maximum antibody concentration, the q_{Ab} was not enhanced at low culture temperature.

To investigate whether lowering the culture temperature affects the transcription level, Northern blot analysis was performed. Since the expression of β-actin mRNA is often considered as a constant and is not affected by temperature (*2*), β-actin mRNA was used as an internal control for Ig mRNA analysis.

Figure 3 shows the Northern blots of Ig and β-actin mRNAs prepared from cell samples during the cultures shown in Figure 1. The relative mRNA content of HC and LC was estimated as a ratio of the mRNA content of HC and LC to β-actin mRNA content for each sample. Like q_{Ab}, the relative HC and LC mRNA was not increased by lowering the culture temperature. In addition, the mRNA level of HC was higher than that of LC regardless of the culture temperature, and we confirmed that the expression of HC was also higher than that of LC by SDS−PAGE analysis (data not shown).

Table 2. Effect of Culture Temperature on q_{Ab}[a]

culture temperature (C°)	q_{Ab} (μg (10^6 cells h)$^{-1}$)	q_{EPO}[b] (μg (10^6 cells h)$^{-1}$)
30	0.44 ± 0.07	0.49 ± 0.14
33	0.50 ± 0.09	0.35 ± 0.08
37	0.43 ± 0.03	0.09 ± 0.03

[a] Values are means ± SD of two independent experiments. [b] q_{EPO} is extracted from ref 6.

The result that low culture temperature did not increase q_{Ab} of rCHO cells (LGA31-56) was rather surprising to us. Arresting cells in G_0/G_1 phase has been suggested as a useful tool for improving production of recombinant proteins. As observed in other rCHO cells (*5*, *6*), the culture of rCHO cells (LGA31-56) at low temperature resulted in rapid accumulation of cells in G_0/G_1 phase. Furthermore, we previously observed that lowering the culture temperature significantly increased specific EPO productivity (q_{EPO}) of rCHO cells (LGE10-9-27) (Table 2) (*6*). The q_{EPO} at 33 °C was approximately 4-fold higher than that at 37 °C. Like q_{EPO}, the relative EPO mRNA content increased by lowering the culture temperature, indicating that the increased transcription level of EPO is responsible in part for the enhanced q_{EPO} at low culture temperature. Both anti-4-1BB antibody and EPO were expressed in the same CHO host and were driven by the same CMV promoters. Therefore, the beneficial effect of lowering the culture temperature on q of rCHO cells appears to be cell-line-specific and may depend on the integration site of a foreign gene. The efficacy of the simultaneous use of hyperosmotic pressure and glycine betaine as a means to improve TPO production in rCHO cells was variable among clones (*14*, *15*). However, clonal variations of rCHO cells in regard to the effect of low culture temperature on q need to be investigated.

In conclusion, cultivation of rCHO cells producing anti-4-1BB antibody (LGA31-56) at low temperature led to suppressed cell growth, cell cycle arrest in G_0/G_1 phase, and improved cell viability for a longer period. However, antibody production and q_{Ab} were not increased at low culture temperature. These results imply that the efficacy of low culture temperature as a means to improve foreign protein production in rCHO cells depends on the cell line.

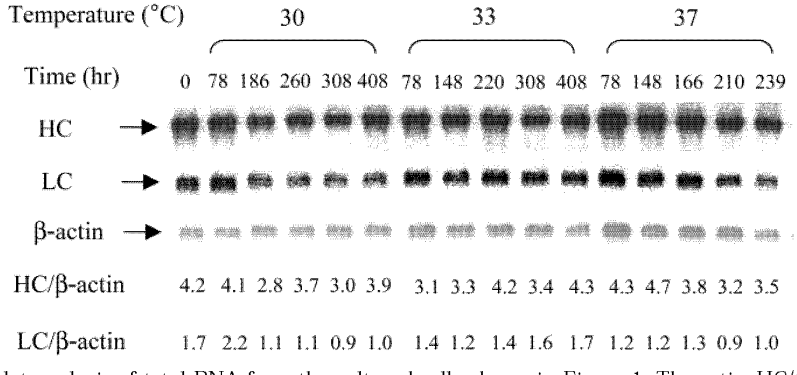

Figure 3. Northern blot analysis of total RNA from the cultured cells shown in Figure 1. The ratio, HC/β-actin and LC/β-actin, shows the relative content of anti-4-1BB mRNA normalized with the internal control, β-actin mRNA.

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Biochemical insights into the mechanisms central to the response of mammalian cells to cold stress and subsequent rewarming

Anne Roobol, Martin J. Carden, Ray J. Newsam and C. Mark Smales

Protein Science Group, Department of Biosciences, University of Kent, Canterbury, UK

Keywords
chaperones; CHO cells; cold stress; cytoskeleton; eIF3i

Correspondence
C. M. Smales, Protein Science Group, Department of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, UK
Fax: +44 01227 763912
Tel: +44 01227 823746
E-mail: c.m.smales@kent.ac.uk

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Mammalian cells cultured *in vitro* are able to recover from cold stress. However, the mechanisms activated during cold stress and recovery are still being determined. We here report the effects of hypothermia on cellular architecture, cell cycle progression, mRNA stability, protein synthesis and degradation in three mammalian cell lines. The cellular structures examined were, in general, well maintained during mild hypothermia (27–32 °C) but became increasingly disrupted at low temperatures (4–10 °C). The degradation rates of all mRNAs and proteins examined were much reduced at 27 °C, and overall protein synthesis rates were gradually reduced with temperature down to 20 °C. Proteins involved in a range of cellular activities were either upregulated or downregulated at 32 and 27 °C during cold stress and recovery. Many of these proteins were molecular chaperones, but they did not include the inducible heat shock protein Hsp72. Further detailed investigation of specific proteins revealed that the responses to cold stress and recovery are at least partially controlled by modulation of p53, Grp75 and eIF3i levels. Furthermore, under conditions of severe cold stress (4 °C), lipid-containing structures were observed that appeared to be in the process of being secreted from the cell that were not observed at less severe cold stress temperatures. Our findings shed light on the mechanisms involved and activated in mammalian cells upon cold stress and recovery.

The heat shock response has been extensively studied in a variety of systems and organisms, and generally involves the conserved and coordinated upregulation of heat shock proteins that act to alleviate the cellular stresses imposed by hyperthermic stress. Our current understanding of the cellular responses to subphysiological temperatures (hypothermia) is less extensive. This is somewhat surprising, because of their relevance in medicine for the storage of cells, organs, and tissues, and the treatment of brain damage; as well as in the biopharmaceutical sector, where reduced culture temperature can sometimes improve recombinant protein yields from mammalian cells cultured *in vitro* [1]. What is clear is that the general response to hypothermia appears to include the global attenuation of transcription and translation, whereas a small group of proteins, termed the cold shock proteins, are selectively induced [2]. However, unlike their heat shock counterparts, these cold shock proteins do not appear to be particularly well conserved between prokaryotic and eukaryotic systems, and their functions, such as have been defined, have to date been described in terms of their RNA rather than their protein biology. Exposure to subphysiological temperature is also known to generally lead to changes in the lipid make-up of membranes, resulting in increased membrane rigidity,

Abbreviations
CCT, chaperonin containing T-complex polypeptide1; Cirp, cold-inducible RNA-binding protein; ER, endoplasmic reticulum; HSF, heat shock factor; NEPHGE, non-equilibrium pH gradient gel electrophoresis; qRT-PCR, quantitative real-time PCR; Rbm3, RNA-binding motif protein 3.

compromised membrane-associated cell functions, and alterations in lipid synthesis and disposition [3].

The most well-characterized cold shock responses to date are those in plants and bacterial systems [3], there being much less information on the molecular mechanisms underpinning the cold shock response in mammalian cells. Our current understanding is that the cold shock response in mammalian cells involves the coordination of transcription, translation, the cell cycle, metabolism, and cell cytoskeleton organization, but the exact mechanisms by which these are modulated remain to be elucidated in most cases. Furthermore, mammalian cells respond to mild hypothermia (25–35 °C) in a different manner to more severely reduced temperatures (0–10 °C). This may largely reflect the fact that at more moderate temperatures, cells can still proliferate and grow, whereas at the severe temperatures, growth is fully arrested. Studies on hibernating animals and various *in vitro* cultured mammalian cell systems have also reported that mammalian cells in general respond to cold shock by disassembly of the cell cytoskeleton, delayed apoptosis, reduced metabolism with reduction of ATP expenditure, reduced protease activity, a reduction in free radical oxygen species, and attenuation of transcription and translation [3]. As a result of such reports, it has been suggested that there are five general mechanisms by which mammalian cells respond to cold shock. These are: (a) a general reduction in transcription/translation; (b) reduction of RNA degradation; (c) increased expression of specific target genes; (d) the generation of alternative mRNAs via presplicing events; and (e) use of internal ribosome entry segments for preferential cap-independent translation of specific mRNAs under cold shock conditions [4].

There have been only two well-characterized mammalian cold shock proteins reported to date, RNA-binding motif protein 3 (Rbm3) [5] and cold-inducible RNA-binding protein (Cirp) [6]. Both of these are induced in response to mild hypothermia (maximal expression around 32 °C) but not severe hypothermia, and are probably general stress response proteins, as they are also induced by a number of other stresses. It is generally thought that Rbm3 and Cirp are involved in the modulation of transcription and translation upon cold stress and function as RNA chaperones, although the exact function of these proteins remains to be elucidated [7]. Cirp and Rbm3 are highly similar proteins that consist of an N-terminal RNA-binding domain and a C-terminal glycine-rich domain, but show no homology to the cold shock proteins found in bacterial systems [3]. Our current understanding of the cold shock response, and the mechanisms involved in coordinating that response, in mammalian cells is therefore extremely limited.

It is interesting to note that although cold stress appears to generally reduce protein synthesis, in recent years recombinant protein production from *in vitro* cultured mammalian cells has been improved by reducing operating temperatures from 37 °C to mildly hypothermic levels (28–34 °C) towards the end of the logarithmic increase in cell number [8]. This strategy has been adopted because although cell division and protein synthesis rates are appreciably slowed, cells show prolonged viability and increased cell-specific productivity under these mildly hypothermic conditions. With regard to the cultivation of mammalian cells at subphysiological temperatures, the prolonged cell viability, delayed apoptosis, decreased glucose and glutamine consumption, decreased waste product release and increased tolerance to shear stress during cultivation under mildly hypothermic conditions are all features likely to extend the productive life of cells in terms of recombinant protein production [1]. However, as the cell division cycle slows and even arrests in G_1 at the lower end of the mildly hypothermic range, and both transcription and translation rates are reduced at subphysiological temperatures, this may offset any potential positive effects of subphysiological culturing.

To date, characterization of the mammalian cold stress response has largely derived from microarray analyses of transcriptional changes and single snapshot proteomic analyses of changes in protein levels upon exposure to subphysiological temperatures [3]. These have been most useful in defining the overall adaptations to growth at subphysiological temperatures and in highlighting areas for further, more detailed investigations into the mechanisms of cold adaptation. Here, we have investigated changes in protein synthesis rates upon cold stress, and show that an examination of changes in the synthesis rates of specific proteins during cold stress, and during recovery, identifies subtle adaptations to growth at subphysiological temperatures, not all of which have been previously uncovered by proteomic analysis of overall protein levels. The proteins identified in this study encompass a wide range of cellular activities, including cell cycle regulation, translation initiation, cytoskeleton organization and, most particularly, molecular chaperone activity. Specific investigation of the roles of a number of the proteins identified leads us to the conclusion that the regulation of p53, Grp75 and eIF3i protein levels may play a key role in the response to, and recovery from, cold stress in mammalian cells. The implications of these findings in terms of the cold stress response in mammalian cells are further discussed.

Results

The cell lines chosen for this study were the commercially relevant CHOK1 cell line and two mouse cell lines, P19 embryonal carcinoma cells and NIH 3T3 fibroblasts. P19 cells were chosen because of their particular sensitivity to cold stress. The temperature ranges investigated covered both severe cold stress (4 and 10 °C) and more mild cold stress (27 and 32 °C), and were considered to be relevant to organ/tissue storage and the subphysiological *in vitro* culturing of mammalian cells for recombinant protein production respectively. The periods of time for which cells were exposed to cold stress (6–30 h) were those sufficient to elicit detectable cold stress responses while still allowing the majority of cells, if not all, to recover upon rewarming.

Severe, but not mild, cold stress results in marked changes in the cellular architecture of *in vitro* cultured mammalian cells

Immunofluorescence studies highlighted changes in the structural architecture of all cell lines investigated upon their exposure to more severe cold stress (Figs 1 and S1). Cells cold-stressed at 27 or 32 °C for 6 h were indistinguishable from those maintained at 37 °C when investigated by immunofluorescence, with the exception that although the microtubule content was unchanged in P19 cells at 27 °C, the microtubule organization appeared to be compromised. Cells cold-stressed at 27 or 32 °C, or maintained at 37 °C, were well spread, and the organelle distribution appeared to be unchanged as exemplified by mitochondrial staining (Fig. 1). Furthermore, when we monitored poly(ADP-ribose) polymerase levels by western blot analysis, there was no evidence of poly(ADP-ribose) polymerase cleavage (Fig. S2A), which is activated upon apoptosis, and therefore we conclude that the cells are not apoptotic at the times and temperatures investigated. At 27 and 32 °C, reduced cell proliferation was observed, and previous reports have suggested that the cold shock protein Cirp may be responsible for cell cycle arrest of mammalian cells at subphysiological temperatures [9]. However, our results suggest that cell cycle arrest at 27 °C is the result of an increase in the overall level (Fig. 2A) and changes to the post-translational modification pattern (Fig. 2B) of p53, which persisted in cells maintained at 27 °C for 6 h to 6 days. The expression of p21, a general inhibitor of cyclin-dependent protein kinases and a downstream effector of elevated p53 levels, was also induced at 27 °C. During recovery from cold stress at 37 °C, the overall amounts of these two proteins, and the isovariants of p53, returned towards their normothermic values and appearance (Fig. 2). The change in isovariant levels of p53 was not due to increased levels of acetylation, as shown by western blot analysis with an antibody specific for acetylated p53 (Fig. S2B). Across the mild cold stress conditions and time periods examined, there was 100% viability and recovery upon returning the cells to 37 °C.

At near-freezing temperatures, the cellular appearance and architecture were profoundly affected, in a cell line-specific manner, as compared with cells maintained at 37 °C. Under conditions of severe cold stress, cells were much more rounded and less well spread, and both nuclear and cellular diameter were decreased. Most cells cold-stressed at 4 °C had translucent, vesicle-like structures that were easily detected by phase contrast viewing, on the surface of the cell (arrowed in Figs 1 and S1). These vesicle-like structures appeared within 2 h of exposure to the severe cell stress but disappeared very rapidly (< 0.5 h) upon rewarming. The content of these vesicles was not revealed by general stains for DNA, RNA or protein, although this last stained the perimeter of the vesicles, but was instead strongly stained by the dye Oil Red O, which preferentially binds to uncharged lipids (Fig. S1). The proportion of cells able to recover from severe cold stress was much smaller than observed for recovery from mild cold stress, and typically 20–30% of cells did not survive upon rewarming from extreme cold (4 °C). Electron microscopy of cold-stressed cells revealed the presence of lipid-containing structures (as stained by osmium tetroxide) corresponding to these vesicles that appeared to be in the process of being extruded from the cell (Fig. 3).

Of the cell lines investigated, CHOK1 cells proved to be the most resilient to subphysiological temperatures, possibly because they contain a sizeable population of cold-stable microtubules and therefore maintain a relatively ordered intracellular organization at low temperatures (Fig. 1). Even at 4 °C, a sizeable population of microtubules persisted in CHO cells, although these appeared to be less ordered than those in cells maintained at 37 °C. Mitotic cells appeared to be particularly vulnerable to cold stress, and below 20 °C cells were observed that contained multiple microtubule asters (Fig. 1; CHOK1 cells at 4 °C, lower section of the upper right panel) that were very similar in appearance to those formed in mitotic cells exposed to the microtubule-stabilizing drug taxol [10]. Such structures are formed when the nuclear envelope breaks down during mitosis and releases proteins (e.g. NuMA) that stabilize microtubule minus-ends and that

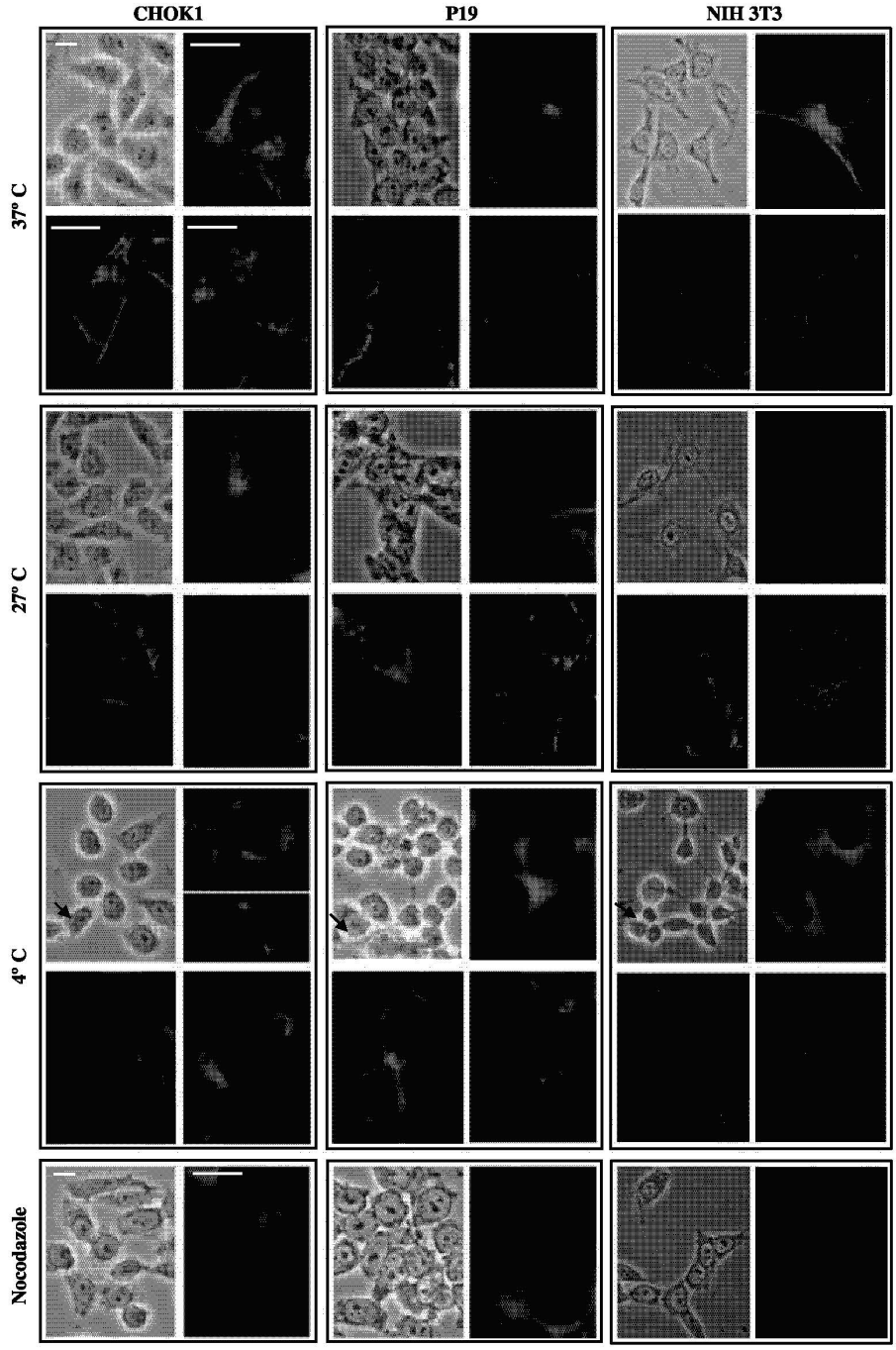

Fig. 1. Structural changes in CHOK1, P19 and NIH 3T3 cells during mild and severe cold stress and after exposure to nocodazole. Cells were maintained at 37 °C, or then transferred to 27 or 4 °C for 6 h, or exposed to growth medium containing 3 $\mu g \cdot mL^{-1}$ nocodazole, prior to fixation for immunofluorescence microscopy. In each panel of four: top left, phase contrast; top right, α-tubulin detection; bottom left, F-actin detection; bottom right, mitochondrial Hsp60 detection. In CHOK1 cells at 4 °C, the lower section of the upper right panel shows the presence of multiple microtubule asters. In each panel of two (nocodazole staining):left, phase contrast; right, α-tubulin detection. Bar: 10 μm. Arrows in the 4 °C phase contrast image point to vesicle-like structures that are also highlighted in Fig. S1.

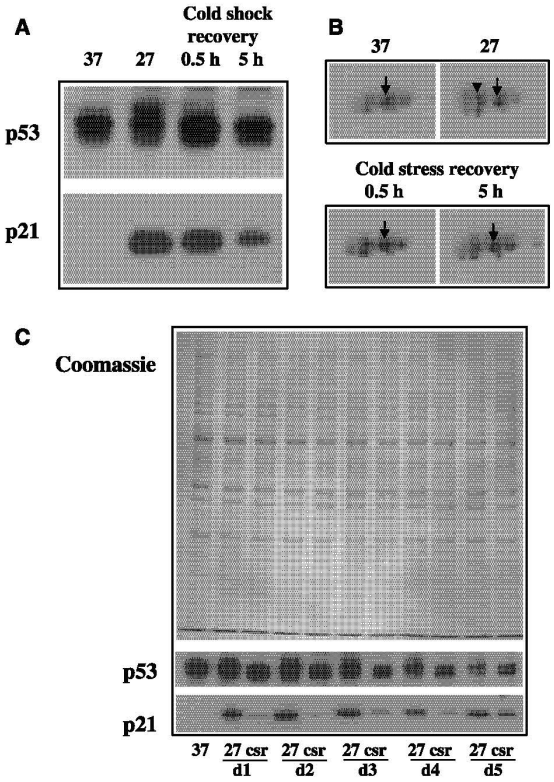

Fig. 2. p53 is elevated and undergoes changes in post-translational modification in CHOK1 cells during maintenance at 27 °C. (A) Twenty micrograms of proteins extracted from CHOK1 cells, maintained at 27 °C or then rewarmed at 37 °C for the indicated times, were resolved by SDS/PAGE and detected by probing immunoblot for p53 or p21. (B) One hundred micrograms of proteins extracted from CHOK1 cells as in (A) were resolved by NEPHGE-SDS/PAGE and immunoblots were probed for p53. Arrows highlight changes in isoform distribution between the 37 and 27 °C samples. (C) As in (A) at 27 °C for up to 5 days (d, day) and upon rewarming (csr, cold shock recovery) for 5 h.

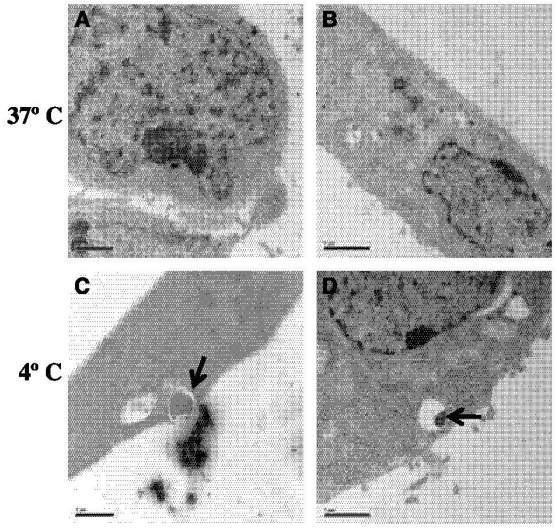

Fig. 3. Electron micrographs of P19 and CHO cells at 37 and 4 °C. P19 cells (A, C) and CHO cells (B, D) at 37 °C or after 6 h at 4 °C. At 4 °C, vesicle-like structures that contain lipids were observed (arrowed) that appeared to be in the process of being extruded from the cell. Bar: 1 μm.

would normally fulfil this function within the mitotic spindle poles. In the presence of taxol, these proteins mis-localize to the cytoplasm, where they stabilize microtubule aster formation [11]. The structures reported here in cold-stressed cells may originate from similar mis-localization of nucleating proteins.

P19 cells had no detectable cold-stable microtubules, and organelle disposition was severely disrupted by temperatures below 20 °C (Fig. 1). The difference in microtubule stability between CHOK1, NIH3T3 and P19 cells was reflected by a relative abundance of STOP proteins (data not shown), splice variants of which stabilize microtubules to cold exposure [12–14]. However, the apparent reduction in cell size and vesicle release observed in cells exposed to severe cold shock is not simply a consequence of a loss of microtubules. Exposure of CHOK1, NIH3T3 and P19 cells to the antimicrotubule drug nocodazole at 37 °C completely depolymerized the microtubule networks in all three cell types and caused retraction of the cytoplasm, but, in contrast to cold-stressed cells, the nuclear size appeared to be unchanged (Fig. 1). Furthermore, no extracellular vesicular structures were observed after nocodazole treatment alone, suggesting that microtubule depolymerization is not the key signal in the formation of the vesicle structures.

Mammalian cells regulate the synthesis rates of specific proteins in response to cold stress and upon recovery at 37 °C

Although there have now been a number of proteomic studies of the cold shock response in various systems that have yielded valuable information, these have been static-based measurement approaches and so do not account for any variation in protein synthesis and turnover rates upon exposure to any given cold-related stress. In order to determine the protein synthesis capacity of *in vitro* cultured mammalian cells at sub-physiological temperatures, newly synthesized proteins were radiolabelled with [^{35}S]methionine/cysteine mix for 1 h at the cold stress temperatures described above.

Scintillation counting was undertaken of samples from cells stressed at different temperatures, to ensure that any difference in the radioactive amino acid uptake did not account for differences in label incorporation into polypeptides (Fig. 4A). The acid-soluble material extracted from CHOK1 cells changed little between 20 and 37 °C, and at 20 °C the acid-soluble/insoluble ratio was actually increased (Fig. 4A). We therefore concluded that methionine/cysteine uptake was not limiting for changes in label incorporated into polypeptide/protein over the temperature range investigated.

SDS/PAGE analysis followed by autoradiography revealed that overall protein synthesis capacity was generally reduced at subphysiological temperatures, although cells at 32 °C, and even at 27 °C, still displayed an appreciable amount of protein synthesis (Fig. 4B), and at this level of resolution the range of proteins being synthesized was similar at 37, 32 and 27 °C. Below 27 °C, protein synthesis was much more severely attenuated, being most affected in the most temperature-sensitive cell line, P19 (Fig. S3), the cell line whose cellular architecture was also most compromised upon severe cold stress. Protein synthesis rates were fully restored upon rewarming to 37 °C, and close examination of the SDS/PAGE analyses revealed that changes in protein synthesis rates were discernible between control cells maintained at 37 °C and those subjected to cold stress and then rewarming, particularly in the 50–75 kDa range (Fig. 4B).

To examine changes in the synthesis rates of individual proteins at subphysiological temperatures and upon recovery more closely, proteins were resolved by 2D non-equilibrium pH gradient gel electrophoresis (NEPHGE)-SDS/PAGE (pI > 4.5 and size 20–150 kDa; Fig. 5A). Owing to this size range limitation, we did not detect the two well-characterized mammalian cold shock-inducible proteins Cirp and Rbm3 (< 20 kDa). However, changes in the synthesis rate (up or down) for 25 newly synthesized polypeptides at 32 °C (Fig. 5B) and for 16 at 27 °C (data not shown) relative to those at 37 °C was observed. The synthesis rates of rather more (31) polypeptides changed upon rewarming after cold stress relative to their rates during continuous growth at 37 °C (Fig. 5C). The synthesis rates of a similar number of polypeptides were observed to change for the other rewarming conditions examined: 4–37 °C (27); 10–37 °C (33); and 20–37 °C (23). The range of polypeptides showing altered synthesis rates during cold shock recovery was similar regardless of whether the recovery was from severe or mild cold stress. Most of the changes in synthesis rates upon cold stress or recovery observed in CHOK1 proteins were observed in similar experiments with P19 and 3T3 cells (Fig. S3).

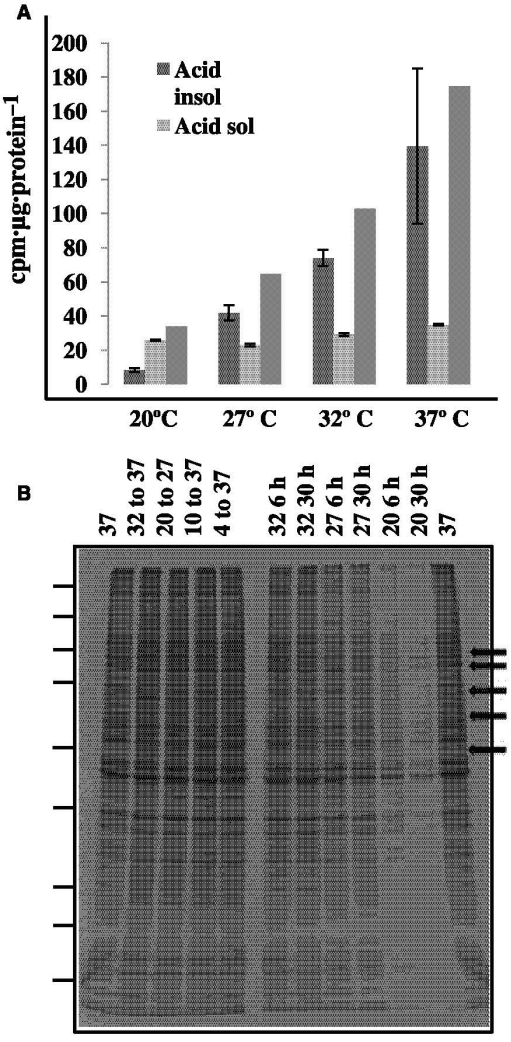

Fig. 4. Amino acid uptake and protein synthesis in CHOK1 cells exposed to, and recovering from, cold stress. (A) Cells maintained at 37 °C, or then exposed to the indicated temperatures for 6 h, were radiolabelled in methionine/cysteine-deficient growth medium supplemented with 1770 kBq·mL^{-1} [^{35}S]methionine/cysteine cell labelling mix for 10 min at the indicated temperature prior to extraction into ice-cold 0.6 M trichloracetic acid. (B) Cells maintained at 37 °C, or then exposed to the indicated temperatures for the indicated times, were radiolabelled as in (A) but for 1 h at the indicated temperatures. Thirty micrograms of extracted proteins were resolved by SDS/PAGE and detected by autoradiography. Molecular mass markers were 205, 150, 100, 75, 50, 37, 25, 20 and 15 kDa, and are indicated by lines on the left-hand side of the figure.

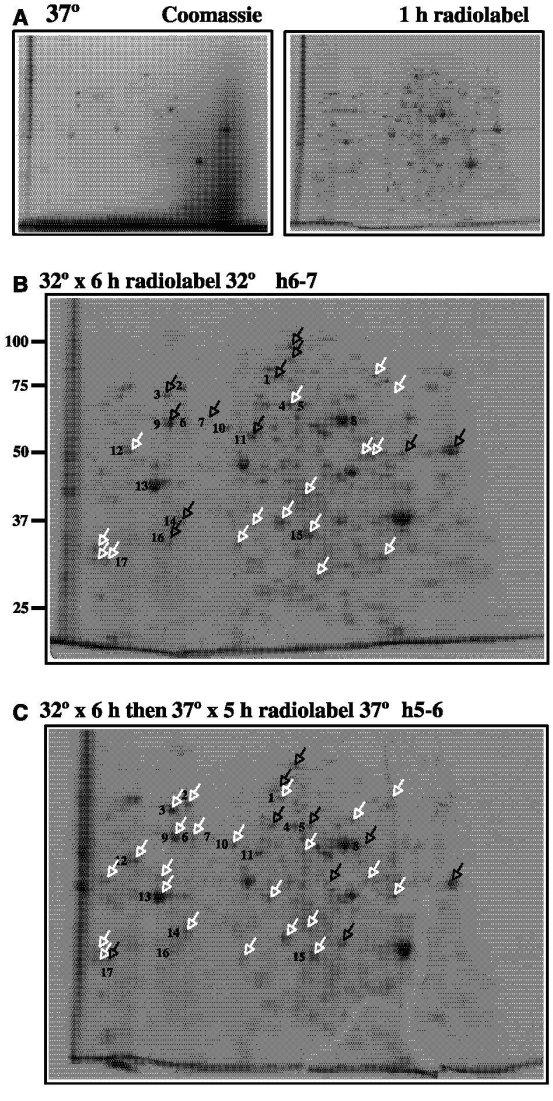

Fig. 5. Changes in protein synthesis rate in CHOK1 cells during exposure to, and recovery from, cold stress. CHOK1 cells were radiolabelled for 1 h under the indicated conditions, and then 100 μg of extracted proteins were resolved by two-dimensional NEPHGE followed by SDS/PAGE. (A) Proteins extracted from cells maintained at 37 °C, detected by Coomassie staining (left panel) or by autoradiography (right panel). (B) Proteins extracted from cells held at 32 °C for 6 h. (C) Proteins extracted from cells held at 32 °C for 6 h and then transferred to 37 °C for 5 h. In (B) and (C), white arrows identify increased synthesis relative to 37 °C, black arrows identify decreased synthesis relative to 37 °C, and numbers identify polypeptides referred to in Table 2.

The more abundant CHOK1 polypeptides (i.e. those readily visible by Coomassie staining) showing altered synthesis rates during cold stress or during recovery were excised and subjected to in-gel tryptic digestion followed by MS analysis by MALDI-TOF MS for their identification. In a number of cases, the identity was confirmed by immunoblot, and in a few cases [subunits of the cytoplasmic molecular chaperone chaperonin containing T-complex polypeptide 1 (CCT)], by a combination of immunoblot and previously identified positions on the NEPHGE-SDS/PAGE system used, and with this approach, 17 CHOK1 polypeptide spots were identified (Table 1). All identified proteins are relatively abundant proteins, but they cover a broad spectrum of functional activities in cells, including energy metabolism, cytoskeleton organization, protein synthesis, protein secretion and purine biosynthesis. The majority, however (9/17), were molecular chaperones deriving from at least three subcellular compartments, the cytoplasm (CCT subunits, Hsc73, and HOP p60), the mitochondrion (Grp75, Hsp60), and the endoplasmic reticulum (ER) (ERp57).

Protein degradation is generally attenuated upon cold stress in mammalian cells

The overall abundance of a polypeptide, and any change in it, depends not only on its rate of synthesis but also its degradation, and so protein degradation rates were also examined at subphysiological temperatures. Examples of the protein half-life determinations for some of the specific CHO proteins investigated are shown in Fig. 6. Interestingly, at subphysiological temperatures relevant to bioprocessing (32 °C), protein degradation was severely curtailed at a global level, being undetectable for all proteins examined at 27 °C over the time period (12 h) investigated. These, remarkably, included the normally very short-lived cell cycle regulator p53. It is notable that the half-life measured for CHO p53 at 37 °C (5.2 h) was longer than reported for this protein in many other cell lines (20–60 min), a fact attributed to the CHOK1 p53 gene having a point mutation in exon 6 sufficient to compromise the normal function; that is, CHOK1 cells fail to arrest in G_1 after radiation-induced DNA damage, and the mutant protein is present at high spontaneous levels in these cells [15].

When the effect of cold stress and recovery on overall protein levels was examined by immunoblot, the changes detected were, with a few exceptions, much more subtle than might have been anticipated from the observed changes in their synthetic rates, particularly as their degradation rates were negligible at 27 °C. This is most likely because the polypeptides chosen for identification were relatively abundant proteins, so that

Table 1. Identification of CHOK1 proteins showing above-average temperature-dependent changes in synthesis rate. NC, no change; ↑, increased abundance; ↓, decreased abundance.

No.	Common name	Method	Identifier, Swissprot	*m* (Da)	pI	Cold stress (°C)		Recovery at 37 °C from (°C):			
						27	32	4	10	20	32
1	NEM-sensitive fusion protein	MS	X15652	83 811	6.38	NC	↓	↑	↑	↑	NC
2	Grp75	MS/blot	U92313	73 970	5.87	NC	NC	↑	NC	↑	↑
3	Hsc73	MS/blot	M34561	70 989	5.24	↑	↓	↑	↑	↑	↑
4	HOP	MS	NM_138911	63 158	6.40	↑	NC	NC	NC	NC	NC
5	IMP cyclohydrolase	MS	D89514	64 705	6.72	NC	NC	↓	NC	NC	↓
6	CCTθ	Blot	Z37164	59 600	5.43	NC	NC	↑	↓	NC	↑
7	CCTα	Blot	M34665	60 339	5.71	NC	↓	NC	NC	NC	NC
8	CCTδ	Blot	Z31554	58 100	8.24	NC	NC	NC	↓	↓	NC
9	Hsp60	MS/blot	M22383	61 122	5.83	NC	↓	↑	NC	NC	↑
10	ERp57	MS	Q91Z81	57 217	5.98	↑	NC	↑	NC	↑	↑
11	CCTβ	MS/blot	Z31553	57 753	5.91	NC	↓	↓	NC	↓	NC
12	β5-Tubulin	MS/blot	NM_011655	50 095	4.78	↑	↑	↑	NC	NC	↑
13	Actin	MS	AB013098	42 087	5.30	↑	NC	↑	↑	↑	↑
14	eIF3i	MS	U39067	36 878	5.38	NC	↓	NC	NC	NC	↑
15	Lactate dehydrogenase A	MS	DQ912661	36 781	7.01	↑	↑	↑	↑	NC	↑
16	β-Tubulin fragment	MS	AJ717320	28 874	4.86	NC	↓	NC	↓	NC	NC
17	Tropomyosin 3	MS	XM_860687	24 918	4.88	NC	↑	NC	NC	NC	↓

a small change in amount due to increased synthesis rate might be difficult to detect by immunoblot against a background of the total polypeptide. Furthermore, the time periods investigated were short (6 h of cold shock, 5 h of recovery), which would make detection of small changes in the total amount of an abundant protein difficult by this method. An exception to this was the θ-subunit of the cytoplasmic chaperonin CCT. The level of this particular subunit was particularly sensitive to both hypothermic and hyperthermic stress (Fig. 7). Comparatively, significant changes in the levels of other subunits of this molecular chaperone were not detectable by immunoblot (data not shown), even though the synthesis rates of several changed during cold shock and/or recovery from cold stress (Table 1).

mRNA degradation is also attenuated during cold stress in mammalian cells

Quantitative real-time PCR (qRT-PCR) was used to ascertain the levels of mRNAs encoding selected proteins for which synthesis rates changed in response to temperature variation. As it was unclear what would be a suitable mRNA to standardize the data to, the data shown in Fig. 8 have been standardized to the respective values at 37 or 27 °C. The mRNAs monitored showed appreciable degradation rates at 37 °C but were found to be more stable at 27 °C. This agrees with a previous study [8] showing increased mRNA levels at reduced temperature, although the study did not analyse synthesis rates. We note further that the more labile a specific mRNA is at 37 °C, the greater are the changes observed in its levels during exposure to, and recovery from, cold stress.

Recovery of cold-stressed cells at 37 °C does not induce a full classical heat shock response

It has been reported that rewarming cold-stressed cells of human origin at 37 °C initiates a heat shock response [16]. Although we also observed that the synthesis rates of several constitutively expressed heat shock proteins increased during recovery from hypothermia, these changes were rather modest when compared with the changes in synthesis rates of these same proteins during recovery from a classical heat shock (hyperthermia; Fig. 9A). Furthermore, upon recovery from heat shock, an increase in the total amount of heat shock proteins could be detected by immunoblot (Fig. 7). Interestingly, Hsp72, which is strictly inducible in rodent cells [17], was not detectable during cold stress or recovery, even though it was clearly induced in the same cells during their recovery from hyperthermic heat shock (Fig. 7, arrowed, Fig. 9A). These observations suggested that the increased expression of constitutive heat shock proteins during recovery from cold stress might be regulated by