late fluids from the surface for long periods.

In our previous examples of a strategy for waste disposal in crystalline rocks, the role of buoyancy-induced flow produced by heat generated in the repository has not been mentioned. This driving mechanism could be significant for inducing flows and transporting nuclear wastes. Recent work (*6*), however, shows that if the waste is allowed to cool for 40 to 60 or 70 years, depending on the waste type, the heat would be reduced to the point where buoyancy-induced flow would not be significant.

Conclusions

The examples presented above illustrate relatively simple alternatives to the current concept of a repository situated

little change in the present approach, additional natural barriers can be brought into play. There are a number of barriers to migration of the wastes: (i) the waste form and its capsule; (ii) engineered barriers within the repository, such as a low-permeability, highly sorptive backfill; and (iii) the migration path back to the biosphere through the ground-water flow system, which in our examples includes flow through the crystalline rocks as well as overlying sedimentary rocks in which sorption could provide yet an additional barrier.

By selecting an environment in which a crystalline rock mass is beneath a sedimentary rock blanket with suitable hydrologic characteristics, one has the advantage that (i) ground-water flow can be investigated with conventional, well-understood technology; (ii) under favorable circumstances, the flow system can

migration and very slow path for the wastes to the biosphere can be assured; and (iii) the wastes can be emplaced in a setting in which the ground water is nonpotable (salty) and not a potentially attractive resource, thus minimizing the possibility of future human intrusion at the site. This is a disposal strategy worthy of careful evaluation.

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Dissections and Reconstructions of Genes and Chromosomes

Paul Berg

The Nobel lecture affords a welcome opportunity to express my gratitude and admiration to the numerous students and colleagues with whom I have worked and shared, alternately, the elation and disappointment of venturing into the unknown. Without their genius, perseverance, and stimulation much of our work would not have flourished. Those who have worked with students and experienced the discomfort of their curiosity, the frustrations of their obstinacy, and the exhilaration of their growth know firsthand the magnitude of their contributions. Each in our common effort left a mark on the other and, I trust, each richer from the experience. I have also been fortunate to have two devoted research assistants, Marianne Dieckmann and June Hoshi, who have labored diligently and effectively, always with understanding and sympathy for my idiosyncracies.

I have also been blessed with an amazing group of colleagues at Stanford University who have created as stimulating and liberating an environment as one could long for. Their many achievements have been inspirational, and without their help—intellectually and materially—my efforts would have been severely handicapped. I am particularly grateful to Arthur Kornberg and Charles Yanofsky, both longtime close personal friends, for their unstinting interest, encouragement, support, and criticism of my work, all of which enabled me to grow and thrive. And finally, there is my wife, Millie, without whom the rare triumphs would have lost their luster. Her strength, assent, and encouragement freed me to immerse myself in research.

Certainly my work could not have taken place without the generous and enlightened support of the U.S. National Institutes of Health, the National Science Foundation, the American Cancer Society, and numerous foundations and individuals who invested their wealth in our research.

> Although we are sure not to know everything and rather likely not to know very much, we can know anything that is known to man, and may, with luck and sweat, even find out some things that have not before been known to man.—J. ROBERT OPPENHEIMER

Although the concept that genes transmit and control hereditary characteristics took hold early in this century, ignorance about the chemical nature of genes forestalled most inquiries into how they function. All of this changed as a result of several dramatic developments during the 1940's to 1960's. First, Beadle and Tatum's researches (*1–3*) lent strong support for earlier (*4*) and widespread speculations that genes control the formation of proteins (enzymes); indeed, the dictum, "one gene–one protein," intensified the search for the chemical definition of a gene. The discovery by Avery and his colleagues (*5*) and subsequently by Hershey and Chase (*6*) that genetic information is encoded in the chemical

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structure of deoxyribonucleic acid (DNA) provided the first clue. Watson and Crick's solution (*7*) of the molecular structure of DNA—the three dimensional arrangement of the polymerized nucleotide subunits—not only revealed the basic design of gene structure but also the outlines of how genes are replicated and function. Suddenly, genes shed their purely conceptual and statistical characterizations and acquired defined chemical identities. Genetic chemistry, or molecular biology as it has frequently been called, was born.

Until a few years ago, much of what was known about the molecular details of gene structure, organization, and function had been learned in studies with prokaryote microorganisms and the viruses that inhabit them, particularly the bacterium *Escherichia coli* and the T and lambdoid bacteriophages. These organisms were the favorites of molecular biologists because they can be propagated readily and rapidly under controllable laboratory conditions. More significantly, utilizing several means of natural genetic exchanges characteristic of these organisms and phages, the mapping and manipulation of their relatively small genomes became routine. As a consequence, discrete DNA molecules, containing one or a few genes, were isolated in sufficient quantity and purity to permit extensive characterizations of their nucleotide sequences and chromosomal organization. Moreover, such isolated genetic elements provided the models, substrates, and reagents needed to investigate a wide range of basic questions: the chemical basis of the genetic code; mutagenesis; the mechanisms of DNA and chromosome replication, repair, and recombination; the details of gene expression and regulation.

The astounding successes in defining the genetic chemistry of prokaryotes during the 1950's and 1960's were both exhilarating and challenging. Not surprisingly, I and others wondered whether the more complex genetic structures of eukaryote organisms, particularly those of mammalian and human cells, were organized and functioned in analogous ways. Specifically, did the requirements of cellular differentiation and intercellular communication, distinctive characteristics of multicellular organisms, require new modes of genome structure, organization, function, and regulation? Were there just variations of the prokaryote theme or wholly new principles waiting to be discovered in explorations of the genetic chemistry o higher organisms? It seemed important to try to find out.

SV40's Minichromosome

Sometime during 1965 to 1966 I became acquainted with Renato Dulbecco's work on the then newly discovered polyoma virus. The growing sophistication of animal cell culture methods had made it possible for Dulbecco's laboratory to monitor and quantify the virus' growth cycle in vitro (*8*). Particularly significant was the discovery that the entire virus genome resided in a single, relatively small, circular DNA molecule, one that could accommodate about five to eight genes (*9*). I was intrigued by the resemblance between polyoma's lifestyles and those of certain bacteriophages. On the one hand, polyoma resembled lytic bacteriophages in that the virus could multiply vegetatively, kill its host, and produce large numbers of virus progeny (*8*). There was also a tantalizing similarity to lysogenic bacteriophages, since some infections yielded tumorigenic cells (*10, 11*). The acquisition of new morphologic and growth characteristics, as well as certain virus-specific properties, suggested that tumorigenesis and cell transformation resulted from covalent integration of viral DNA into the cell's chromosomal DNA and the consequent perturbation of cell growth control by the expression of virus genes (*12, 13*).

These discoveries and provocative speculations, together with an eagerness to find an experimental model with which to study the mechanisms of mammalian gene expression and regulation, prompted me to spend a year's sabbatical leave (1967 to 1968) in Dulbecco's laboratory at the Salk Institute. The work and valuable discussions we carried on during that time (*14*) reinforced my conviction that the tumor virus system would reveal interesting features about mammalian genetic chemistry.

For somewhat technical reasons when I returned to Stanford, I adopted SV40, a related virus, to begin our own research program. SV40 virions are nearly spherical particles whose capsomers are organized in icosahedral symmetry (Fig. 1a). The virions contain three viral coded polypeptides and a single double-stranded circular DNA molecule (Fig. 1b), that is normally associated with four histones, H2a, H2b, H3, and H4 in the form of condensed (Fig. 1c) or beaded (Fig. 1d) chromatin-like structures. The SV40 DNA contains 5243 nucleotide pairs [5.24 kilobase pairs (kbp)], the entire sequence of which is known from studies in the laboratories of S. Weissman (*15*) and W. Fiers (*16*). Coding information for five (and possibly six) proteins is contained in the DNA nucleotide sequence. Three of the proteins occur in mature virions, possibly as structural components of the capsid shell, although one might be associated with the DNA and have a regulatory function (*17*). Of the two nonvirion proteins encoded in the DNA sequence, one is localized in the cell nucleus (large T antigen) and functions in viral DNA replication and cell transformation; the other, found in the cytoplasm (small t antigen), enhances the efficiency of cell transformation (*18*). Other proteins related in structure to large T antigen have been speculated about but their structures and functions are unclear.

Restriction endonucleases have played a crucial role in defining the physical and genetic organization of the SV40 genome (*19, 20*). The restriction or cleavage sites served as coordinates for a physical map of the viral DNA; the availability of such map coordinates made it possible to locate, accurately, particular physical features and genetic loci. In this system of map coordinates, the single Eco RI endonuclease cleavage site serves as the reference marker and is assigned map position 0/1.0; other positions in the DNA are assigned coordinates in DNA fractional length units measured clockwise from 0/1.0 (see Fig. 2). At the present time, knowledge of the entire nucleotide sequence has made possible a more precise set of map coordinates: nucleotide pair number. Thus, nucleotide 0/5243 is placed within ori, the site where DNA replication is initiated, and the other nucleotide pairs are numbered consecutively in the clockwise direction (see Fig. 2).

The SV40 minichromosome is expressed in a regulated temporal sequence after it reaches the nucleus of infected primate cells. Initially, transcription in the counterclockwise direction of one strand (the E strand) of about one-half of the DNA (the early region) yields the early messenger RNA's (mRNA's) (Fig. 2). These mRNA's, which encode the large T and small t antigen polypeptides (the stippled portion of the mRNA's indicate the protein coding regions), have 5′ ends originating from nucleotide sequences near the site marked ori, and 3′-polyadenylated [poly(A)] ends from near map position 0.16. Synthesis of large T antigen triggers the initiation of viral DNA replication at ori, a specific site in the DNA (Fig. 2 identifies ori at map position 0.67 or nucleotide position 0/5243); replication then proceeds bidirectionally, terminating about 180° away near map position 0.17, yielding covalently closed circular progeny DNA. New viral mRNA's appear in the polyribosomes concomitantly with DNA replication; these are synthesized in the clockwise direction from the L strand of the other half of the virus DNA (the late region) and are referred to as late mRNA's. Transcription of the late mRNA's, which code for the virion proteins VP1, VP2, and VP3 (the stippled regions designate the protein coding regions of these proteins), begins from multiple positions between map positions 0.68 to 0.72 and terminates at about map position 0.16. Finally, the accumulation of progeny DNA molecules and virion proteins culminates in death of the cell and release of mature virion particles.

SV40 possesses an alternative life cycle when the virus infects rodent and other nonprimate cells. The same early events take place—the E strand mRNA's and large T and small t antigens are synthesized—but DNA replication does not occur, and late strand mRNA's and virion proteins are not made. Frequently, replication of cell DNA and mitosis are induced after infection, and most infected cells survive with little evidence of prior infection. Generally, a small proportion of the cells (less than 10 percent) acquire the ability to multiply under culture conditions that restrict the growth of normal cells; moreover, these transformed cells can produce tumors after inoculation into appropriate animals. Invariably, the transformed cells contain all or part of the viral DNA covalently integrated into the cell's chromosomal DNA and produce the mRNA's and proteins coded by the early genes.

During the 1970's several different approaches, carried on in many laboratories including my own, clarified the arrangement of SV40's genes on the DNA and revealed how they function during the virus' life cycle (*21–23*). Initially, viral genes were mapped on the DNA relative to restriction sites by localizing the regions from which early and late mRNA's were transcribed. Subsequently, more precise mapping was achieved by correlating the positions of discrete deletions and other alterations in the viral DNA with specific physiologic defects. But with the nucleotide sequence map, the boundaries of each SV40 gene and the nucleotide segments coding for each polypeptide can be specified with considerable precision (Fig. 2). As expected, the availability of a precise genetic and physical map of SV40's minichromosome has shifted the research emphasis to explorations of the molecular mechanisms governing each gene's expression and function, the replication and maturation of the viral minichromosome, recombination between the viral and host DNA, and how virus and host gene products interact to cause transformation of normal into tumorigenic cells. Excellent and more detailed summaries and analyses of the molecular biology of SV40 and polyoma, containing acknowledgments to the important contributions made by many individuals, can be found in several recent monographs (*21–23*).

SV40 as a Transducing Virus

The analysis of the organization, expression, and regulation of bacterial genomes was greatly aided by the use of bacteriophage-mediated transfer of genes between cells. Indeed, specialized transducing phages of λ, ϕ80, P22, and others permitted the cloning and amplification of specific segments of bacterial DNA, thereby making it possible to construct cells with unusual and informative genotypes and to obtain valuable substrates and probes for exploring mechanisms of transcription, translation, and regulation.

This background led me to consider, soon after beginning work with the tumor viruses, whether SV40 could be used to transduce new genes into mam-

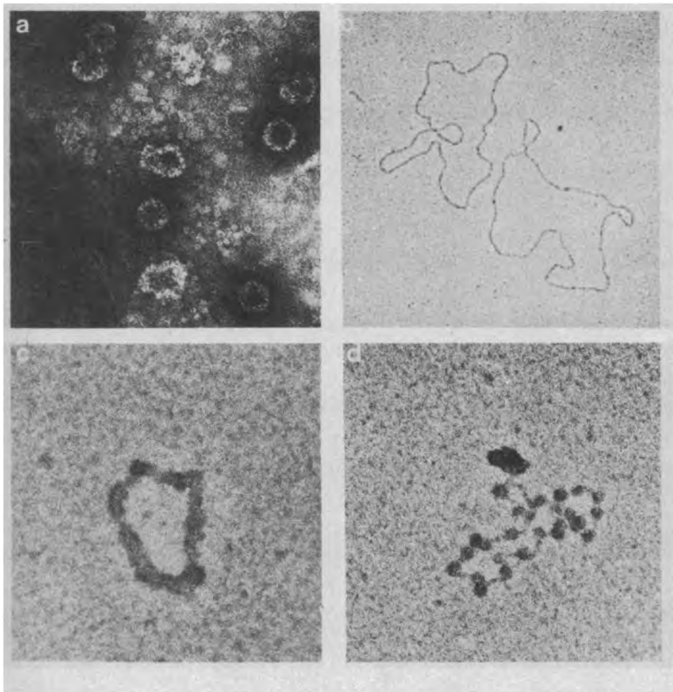

Fig. 1 (left). Electron micrographs of: (a) SV40 virions; (b) SV40 DNA; (c) "condensed" SV40 minichromosomes; (d) "relaxed-beaded" SV40 minichromosome. [Photo by J. Griffith]

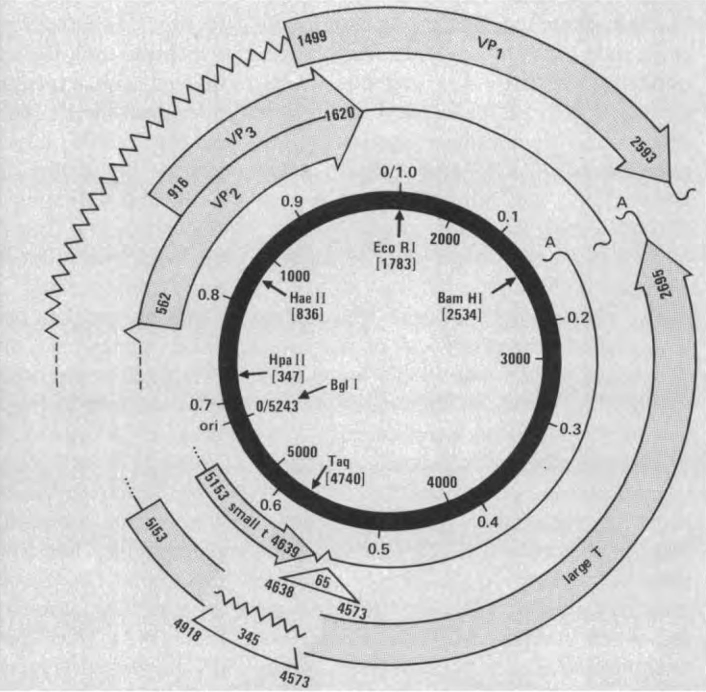

Fig. 2 (right). A physical and genetic map of SV40 DNA. The inner circle symbolizes the closed circular DNA molecule; indicated within the circle are the nucleotide-pair map coordinates starting and ending at 0/5243. Also shown by small arrows within the circle are the sites at which five restriction endonucleases cleave SV40 DNA once. Arrayed around the outside of the circle are the map coordinates, expressed in fractional lengths, beginning at the reference point 0/1.0 (the Eco RI endonuclease cleavage site) and proceeding clockwise around the circle. The coding regions for the early and late proteins are shown as stippled arrows extending from the nucleotide pair of the first codon to the nucleotide pair that specifies termination of the protein coding sequence. Each of the coding regions is embedded in an mRNA, the span of which is indicated by dotted or dashed 5′ ends and wavy poly(A) 3′ ends. The jagged or saw-toothed portions of each mRNA indicate the portions of the transcript that are spliced in forming the mature mRNA's.

malian cells. Initially, I had serious reservations about the success of such a venture because of the predictably low probability of generating specific recombinants between virus and cell DNA and the limited capability for selecting or screening animal cells that had acquired specific genetic properties. But it seemed that one possible way out of this difficulty, at least one worth trying, was to produce the desired SV40 transducing genomes synthetically. Consequently, in about 1970, I began to plan the construction in vitro of recombinant DNA molecules with SV40 and selected nonviral DNA segments. The goal was to propagate such recombinant genomes in suitable animal cells, either as autonomously replicating or integrated DNA molecules. At the time there were few if any animal genes available for recombination with SV40 DNA, but I anticipated that a variety of suitable genes would eventually be isolated. Therefore, the first task was to devise a general way to join together in vitro any two different DNA molecules.

Hershey and his colleagues had already shown that λ phage DNA could be circularized or joined end to end in vitro (*24*). This occurred because λ phage DNA has cohesive ends, that is, single-stranded, overlapping, complementary DNA ends (*25*). So, it seemed that if cohesive ends could be synthesized onto the ends of DNA molecules, they could be covalently joined in vitro with DNA ligase.

During 1971–1972, using then available enzymes and relatively straightforward enzymologic procedures, David A. Jackson, Robert H. Symons, and I (*26*) and, independently and concurrently, Peter E. Lobban and A. D. Kaiser (*27*), devised a way to synthesize synthetic cohesive termini on the ends of any DNA molecules, thereby paving the way for constructing recombinant DNA's in vitro. We developed a procedure (Fig. 3) using as the model "foreign" DNA, a bacterial plasmid that contained some bacteriophage λ DNA and three *E. coli* genes that specify enzymes required for galactose utilization (*28*). Circular SV40 DNA (5.24 kbp) and λdv gal plasmid DNA (about 10 kbp) were each cleaved with a specific endonuclease to convert them to linear molecules. Then, after a brief digestion with λ exonuclease to remove about 50 nucleotides from the 5′ termini, it was possible for deoxynucleotidyl terminal transferase to add short "tails" of either deoxyadenylate or deoxythymidylate residues to the 3′ termini. After mixing and annealing under appropriate conditions, the two DNA's were joined and cyclized via their complementary "tails" (Fig. 3). The gaps that occur where the two DNA molecules are held together, were filled in with DNA polymerase I and deoxynucleoside triphosphate substrates, and the resulting molecules were covalently sealed with DNA ligase; exonuclease III was present to permit repair of nicks or gaps created during the manipulations.

The resulting hybrid DNA was approximately three times the size of SV40 DNA and, therefore, could not be propagated as a chromosome in a virus capsid. But we intended to test whether the *E. coli* galactose genes would be expressed after introduction into the chromosomes of cultured animal cells. Moreover, since the λdv gal plasmid could replicate autonomously in *E. coli* (*28*), we also planned to determine whether SV40 DNA would be propagated in *E. coli* cells and whether any SV40 genes would be expressed in the bacterial host. Although the SV40-λdv gal recombinant DNA shown in Fig. 3 could not have replicated in *E. coli*—a gene needed for replication of the plasmid DNA in *E. coli* had been inactivated by the insertion of the SV40 DNA—a relatively simple modification of the procedure, namely, the use of λdv gal dimeric DNA as acceptor for the SV40 DNA insert, could have circumvented this difficulty. Nevertheless, because many colleagues expressed concern about the potential risks of disseminating *E. coli* containing SV40 oncogenes, the experiments with this recombinant DNA were discontinued.

Since that time there has been an explosive growth in the application of recombinant DNA methods for a number of novel purposes and challenging problems. This impressive progress owes much of its impetus to the growing sophistication about the properties and use of restriction endonucleases, the development of easier ways of recombining different DNA molecules, and, most importantly, the availability of plasmids and phages that made it possible to propagate and amplify recombinant DNA's in a variety of microbial hosts [see (*29*, *30*) for a collection of notable examples].

By 1975, extensive cloning experiments had produced elaborate libraries of eukaryote DNA segments containing single genes or clusters of genes from many species of organisms. As expected, studies of their molecular anatomy and chromosomal arrangement have provided new insights about possible mechanisms of gene regulation in normal and developmentally interesting animal systems. But, it seemed likely from the beginning that ways would be needed to assay isolated genes for their biological activity in vivo. Consequently, I returned to the original goal of using SV40 to introduce cloned genes into cultured mammalian cells. But this time we explored a somewhat different approach.

During 1972 to 1974 Janet Mertz and I (*31*) learned how to propagate SV40 deletion mutants by complementation, using appropriate SV40 temperature-sensitive (ts) mutants as helpers. This advance made it feasible to consider propagating genomes containing exogenous DNA in place of specific regions of SV40 DNA.

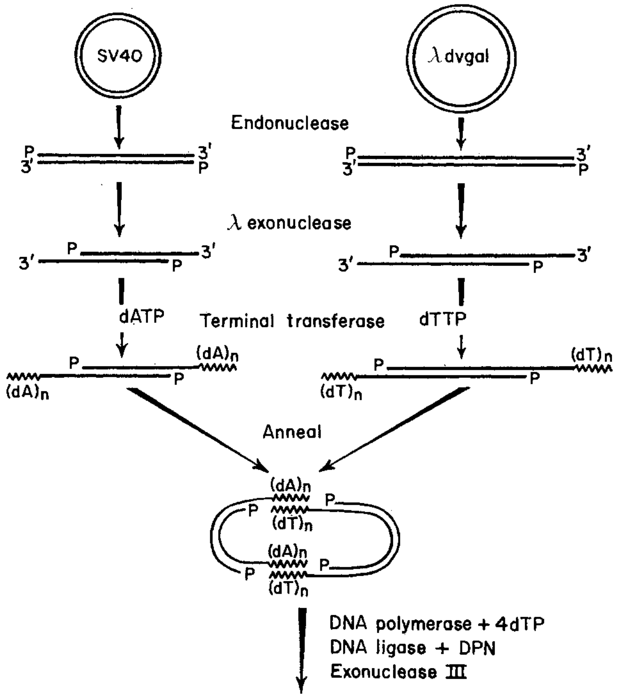

Fig. 3. The construction of SV40-λdvgal recombinant DNA. See text for comment on individual steps. *4dTP*, 4 deoxy triphosphates; *DPN*, nicotinamide adenine dinucleotide.

Accordingly, Stephen Goff and I devised a procedure to construct such recombinants by removing defined segments of SV40 DNA with appropriate restriction endonucleases and replacing them with foreign DNA segments, using synthetic cohesive ends (*32, 33*) (Fig. 4). In this experimental design the recombinant genomes must contain the origin of SV40 DNA replication (ori) so that they can be propagated; also, they must be smaller than 5.3 kbp, that is, not more than one mature viral DNA length, to be incorporated into virus particles. Furthermore, because the SV40 vector lacks genetic functions coded by the excised DNA segment, the recombinant genomes are defective and must be propagated with a helper virus that can supply the missing gene product or products. In our protocol, the recombinant genome retains at least one functioning virus gene and, consequently, can complement a defective gene in the helper virus. For example, recombinants in which the inserted DNA replaces all or part of SV40's late region can be propagated with SV40 mutants that have a defective early region (for example, at high temperature with ts early mutants); similarly, recombinants having exogenous DNA implants in place of DNA segments in the early region can be propagated with a helper genome that is defective in its late region (in this instance with ts late mutants).

Our initial attempts (*32, 33*) to obtain expression of cloned segments as distinct mRNA's and proteins, following the introduction of the recombinant genomes into cultured cells, were negative. But as soon as we recognized that expression of the new genetic information required that the transcript, originating from SV40 promoters and ending at SV40-specified poly(A) sites, be spliced, our fortunes changed. The initial success in obtaining expression of added genetic elements as mRNA's and proteins after transfection into cultured monkey cells was achieved with a DNA segment coding for rabbit β-globin (*34*). Soon afterward, a bacterial gene (Ecogpt) coding for xanthine-guanine phosphoribosyl transferase (XGPRT) (*35*), a mouse DNA specifying dihydrofolate reductase (DHFR) (*36*), and a bacterial gene (neo^R) for aminoglycoside phosphotransferase (*37*) were successfully transduced into mammalian cells via SV40 DNA based vectors. Generally, the transduced DNA segments are expressed at rates comparable to those of the SV40 genes they replace, but some anomalies in the RNA processing have been observed.

Hamer and Leder have also constructed and propagated recombinants of SV40 DNA with cloned mouse genomic β-globin (*38*) or α-globin (*39*) genes. In certain of their recombinants the transduced genes are expressed from SV40 late promoter signals, but other constructions reveal that transcription can be initiated from the α-globin promoter as well (*39*). Their experiments also demonstrate that proper splicing of the globin intervening sequences and translation of the resulting mRNA's can occur in a heterologous host.

New Transducing Vectors for Mammalian Cells

In the experiments referred to above, our principal aim had been to exploit the ability of the recombinant genomes to replicate in the virus' permissive host. For example, following infection of monkey cells, the SV40 recombinant genomes are amplified about 10^4- to 10^5-fold, thereby ensuring a high yield of the products expressed from the transduced genes. This system has taught us a great deal about the necessity and mechanistic subtleties of RNA splicing (*40*), the rules governing expression of coding sequences inserted at different positions in SV40 DNA (*41*), and some novel features of SV40 gene expression itself (*42*). But this experimental design has several distinct shortcomings. During the course of the infection the cells are killed, precluding the opportunity to monitor the transduced gene's expression in continuously multiplying cell populations. Moreover, only cells which can replicate SV40 DNA are able to amplify the cotransduced genes. This constraint excludes many specialized and differentiated animal cells as hosts for the transduced genes.

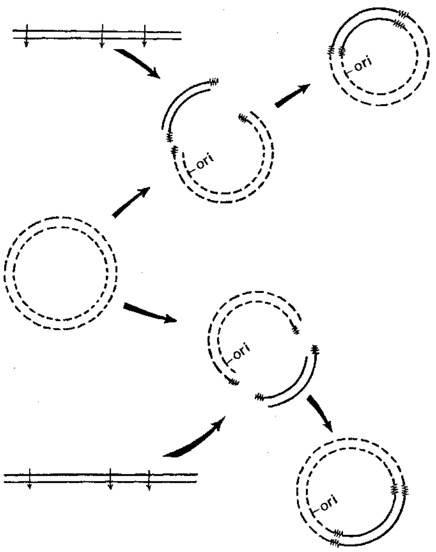

Fig. 4. A scheme for constructing SV40 transducing genomes in vitro. Segments of the late (upward track) or early (downward track) regions of SV40 DNA (the dashed circle on the left) are removed by sequential cleavages with restriction endonucleases. Appropriate-sized segments of any DNA, produced by restriction enzyme cleavages, enzymatic copying of mRNA, or chemical synthesis, are inserted in place of the resected SV40 DNA segment. Joining, via natural or synthetic cohesive ends (symbolized by the jagged lines), is mediated by a DNA ligase. *ori* indicates the position of the origin of SV40 DNA replication.

To circumvent these disadvantages, we have developed a new group of transducing vectors that can be used to introduce and maintain new genetic information in a variety of mammalian cells (Fig. 5). pSV2 (*43*), and its derivatives pSV3 and pSV5 (*35, 44*) contain a DNA segment (shown as the filled region in Fig. 5) from an *E. coli* plasmid (pBR322) that permits these DNA's to propagate in *E. coli* cells, thereby greatly simplifying the genetic manipulations involved in their use. Each of the vectors contains a marker gene (shown in Fig. 5 as the hatched segment) flanked at the 5′ end with a DNA segment containing the SV40 early promoter and origin of DNA replication (ori); another SV40 DNA segment that ensures splicing and polyadenylation of the transcript is located at the 3′ end of the marker segment (the SV40-derived DNA segments are shown stippled in Fig. 5). Additional DNA segments can also be inserted into the vector DNA's at any of several unique restriction sites; consequently, a single DNA molecule can transduce several genes of interest simultaneously.

pSV2 cannot replicate in mammalian cells because it and the cell lack the means to initiate DNA replication at ori. This can be rectified by inserting, at pSV2's single Bam HI cleavage site, DNA segments which contain either a complete early region from SV40 DNA (pSV3), or polyoma's early region (pSV5) (Fig. 5). The viral early regions inserted into pSV3 and pSV5 vectors code for proteins that promote DNA replications from their respective origins, therefore pSV3 DNA can replicate in monkey cells and pSV5 DNA replicates in mouse cells (*44*).

To date, three marker DNA segments have been used in conjunction with the pSV2, pSV3, and pSV5 vector DNA's: Ecogpt, an *E. coli* gene that codes for the enzyme XGPRT (*35, 44*); a mouse complementary DNA (cDNA) segment that specifies DHFR (*36*); neo^R, a bacterial plasmid gene specifying an aminoglycoside phosphotransferase that inactivates the antibacterial action of neomycin-

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