Ann. Hum. Genet. (1981), **45**, 135–141
Printed in Great Britain

Chromosomal localization of a single copy gene by *in situ* hybridization – human β globin genes on the short arm of chromosome 11

By S. MALCOLM, P. BARTON, C. MURPHY and M. A. FERGUSON-SMITH
Biochemistry Department, Queen Elizabeth College, London, W8, and Institute of Medical Genetics, Yorkhill, Glasgow

SUMMARY

1. The localization of the β globin genes by *in situ* hybridization to fixed chromosomes is described.

2. The probe used was a [^{3}H]cRNA copy of a genomic clone containing in total 4·4 kb of DNA and including the β globin gene.

3. The evidence for the localization of the gene comes from three pieces of data. (*a*) Chromosome 11 is labelled to double the extent expected if the grains were randomly distributed, (*b*) the extra grains above background are clustered on the short arm of 11 close to the centromere, and (*c*) the absolute number of grains observed is very close to that predicted for a probe of that length by comparison with ribosomal genes. The localization is in agreement with that obtained by other methods.

4. This method could be extended to any gene for which a genomic clone containing at least 5 kb of single copy DNA is available.

INTRODUCTION

In situ hybridization to fixed metaphase preparations from cultured lymphocytes has been used to locate several repetitive gene families in man such as 28S and 18S ribosomal RNA genes (Evans, Buckland & Pardue, 1974), 5S genes (Steffensen *et al.* 1975) and histone genes (Chandler *et al.* 1979). This method of gene localization can be used directly on normal chromosome preparations and avoids the problems associated with somatic cell hybrids which are time-consuming to prepare and may contain undetected chromosomal rearrangements or fragments. The use of chromosomal translocations allows very precise localizations to be made (Fennell *et al.* 1979).

The method has previously been restricted to repetitive genes because of the extremely small quantity of DNA found in a single chromosome and the maximum possible specific radioactivity of the RNA or DNA used as a gene probe in hybridization. However, the availability of cloned genomic fragments containing structural genes and the intervening and surrounding sequences (Lawn *et al.* 1978) means that the length of DNA sequence hybridized for single copy genes may approach that for repetitive families. We have used an RNA transcript from a restriction fragment from one of the best characterized systems, the β globin gene, to confirm both the localization of this gene on chromosome 11 and the regional localization close to the centromere on the short arm.

0003-4800/81/0000-4516 $01.00

METHODS

Chromosome preparations

Metaphase chromosomes were prepared by culturing normal lymphocytes with phytohaemagglutinin as previously described (Ferguson-Smith, 1974). Before hybridization chromosomes were treated with a 1 % aqueous solution of lipsol detergent and stained with Leischman's stain for photography.

In situ *hybridization*

After treatment with RNAase A (Sigma) at 100 μg/ml at 37 °C for 30 min, chromosomes were denatured in 60 % formamide, 5 mM HEPES, 1 mM EDTA, pH 7·0, at 55 °C for 15 min. Ten ng of [^{3}H]cRNA (specific activity $1{\cdot}8 \times 10^8$ dpm/μg) in 5 μl of hybridization buffer (50 % formamide, 0·6 M-NaCl, 5 mM HEPES pH 7·0) were incubated with each slide at 43 °C overnight. Slides were then treated with RNAase A at 10 μg/ml, 37 °C, 30 min to remove non-hybridized or mismatched labelled cRNA and washed thoroughly in 2 × SSC. After dehydration through a series of alcohols, the slides were dipped in Ilford K2 Nuclear Emulsion and exposed at 4 °C for 40 days. The slides were then developed and the cells that had previously been photographed were re-examined with a microscope for silver grains.

Complementary RNA. [^{3}H]cRNA was made by the method of Jones (1974). ATP, CTP and UTP were radioactively labelled with [^{3}H] and the specific activity of the cRNA was $1{\cdot}7 \times 10^8$ dpm/μg.

RESULTS

Hybridization probe

The recombinant plasmid (HβIS) used to prepare the cRNA gene probe contains the human β globin gene and its intervening and surrounding DNA sequences to a total length of 4·4 kilobases (kb). This plasmid, kindly provided by Dr Tom Maniatis of California Institute of Technology, was prepared by inserting a Pst I restriction fragment containing the β globin genes into plasmid PBR 322. A full restriction map of this region of the chromosome is shown in Bernards *et al*. (1979). This fragment hybridizes to a single band of restricted DNA in Southern blot hybridization at a final wash of 0·1 × SSC, 65 °C (T. Maniatis, personal communication, and our unpublished data).

Chromosomal distribution of grains

Human metaphase cells were banded and stained with Lipsol/Leischman's and photographed. The position of each cell was noted and, after *in situ* hybridization and autoradiography, the same cells were relocated by microscope and scored for autoradiographic silver grains. This allows the unequivocal identification of each chromosome. Fifty-five cells were karyotyped and the grains falling on each chromosome determined (Table 1). Since the cells have been selected for the quality of the metaphase preparation prior to *in situ* hybridization, observer bias in cell selection cannot occur. An allowance for the different lengths of the chromosomes has been made by dividing the number of grains occurring over each chromosome by the percentage of the total genomic DNA found in that chromosome (Ferguson-Smith, 1974). This gives the number of grains per unit length for each chromosome where a unit length is 1 % of the DNA of the human

Table 1. *Total grain counts per chromosome in 55 cells analysed*

Chromosome	Total no. of grains	% of genome	Grains/unit length
1	72	8·47	8·50
2	61	7·76	7·86
3	63	6·56	9·60
4	54	6·13	8·81
5	58	5·58	10·39
6	51	5·65	9·03
7	42	5·00	8·40
8	43	4·77	9·01
9	51	4·73	10·78
10	47	4·35	10·80
11	93	4·35	21·38
12	53	4·16	12·74
13	34	3·59	9·47
14	36	3·28	10·97
15	37	3·11	11·89
16	49	3·11	15·75
17	31	3·02	10·26
18	38	2·73	13·92
19	26	2·58	10·08
20	21	2·31	9·09
21	12	1·90	6·31
22	22	1·69	13·01
X	23	5·14	8·94
Y	8	2·09	7·65
			$\bar{x} = 10{\cdot}61$
			$\sigma = 3{\cdot}06$

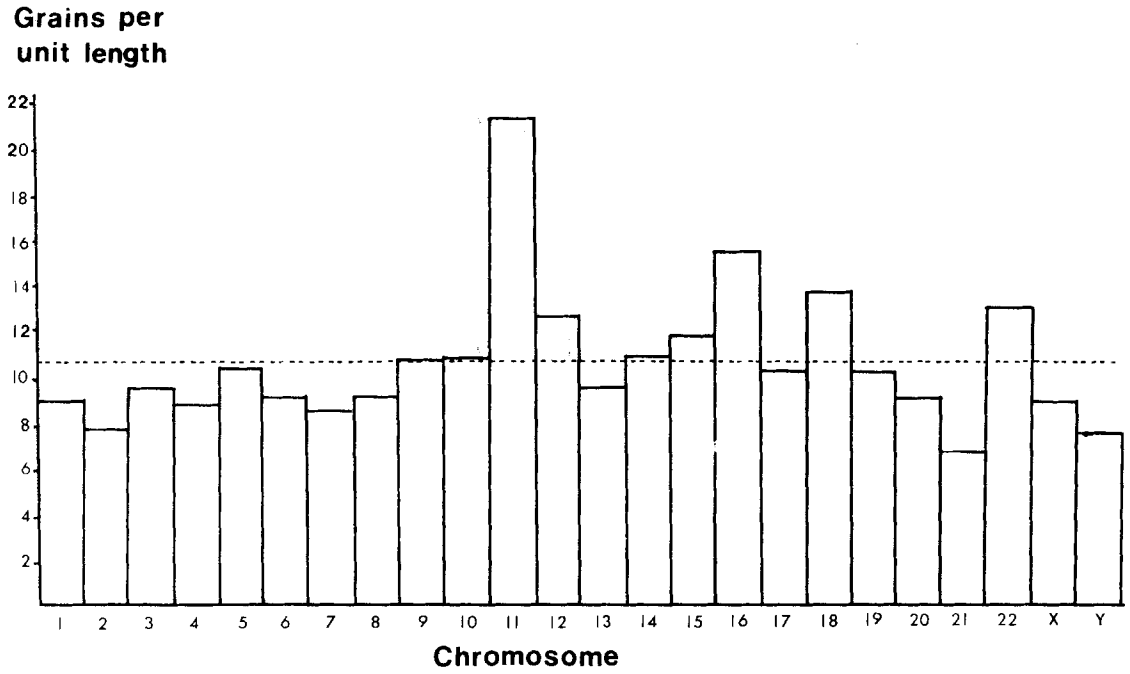

Fig. 1. Distribution of grains per unit chromosome length (1 % of the genome). The dotted line indicates the grains expected if grains are randomly distributed.

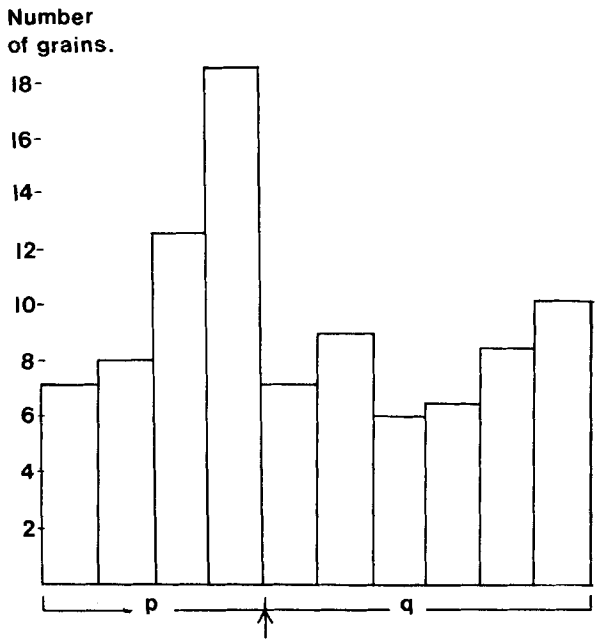

Fig. 2. Distribution of grains along chromosome 11. The arrow indicates the position of the centromere. p and q represent short and long arms respectively. The chromosome was divided into ten equal-sized segments.

diploid genome (Fig. 1). A calculation of the grains/unit length expected if all the observed grains were due to random background has been carried out by dividing the total number of grains by the total number of length units. This value is shown as a dotted line in Fig. 1.

Chromosome 11 shows twice as much hybridization per unit length as would be expected if all the grains were distributed randomly (see Fig. 1) and no other chromosome shows anything approaching this level of hybridization. When the grains/unit length for all the chromosomes are analysed the value for chromosome 11 falls outside 3·5 standard deviations from the average (see Table 1), making it extremely unlikely that this is a chance value. No other chromosome falls significantly away from the mean although chromosome 16 shows hybridization somewhat above background and is the next most heavily labelled chromosome. As expected, the smaller chromosomes, which represent a smaller proportion of the total counts, differ more from the average than the larger chromosomes.

Distribution of silver grains along chromosome 11

The distribution of silver grains along the length of chromosome 11 was analysed and is shown in Fig. 2. The chromosome was arbitrarily divided into ten equal units and these units merely represent the resolution with which we are able to assign grains and do not represent cytogenetic bands. Fig. 2 shows that there is a marked concentration of grains adjacent to the centromere in the short (p) arm of the chromosome. All other parts of chromosome 11 show hybridization close to the background level expected for random distribution.

Efficiency of hybridization

It is possible to make an estimate of the number of grains that would be expected after 40 days exposure for a locus of 4·4 kb present as a single copy and labelled to the specific activity used by comparison with known grain counts for the 18S and 28S ribosomal RNA genes in man. The ribosomal genes are distributed amongst five chromosomes in man, and as there are approximately 50 copies of the ribosomal genes per haploid genome (Young, Hell & Birnie, 1976) on average there will be ten copies per chromosomal nucleolar organizer locus, or 70 kb DNA sequence. In control experiments we find 1–2 grains per nucleolar organizer after 1 week's exposure for this specific activity cRNA probe from a ribosomal gene recombinant. For the globin gene sequence 4·4 kb in length, we would expect approximately 0·5 grains per chromosomal locus after 40 days exposure. Since a total of 55 cells (110 chromosome 11 s) were scored, a total of 55 grains above background would be expected by comparison with the ribosomal gene locus. Using the background calculated from all other chromosomes, there is an excess of 47 grains over chromosome 11 in the total number of 55 cells, in close agreement with the predicted value.

DISCUSSION

Human ribosomal genes may be readily detected using short exposure times by *in situ* hybridization and this has proved useful for gene localization and the study of variants (Salmasi *et al.* 1980; Elliott *et al.* 1980). As an NOR contains on average 70 kb of ribosomal DNA and the average mRNA only 1 kb or less it seemed that this method would not be generally applicable to the localization of single copy structural genes. It could be calculated that about 5 kb of hybridizing sequence would give on average 0·5 grains/chromosome under the conditions of overall efficiency achieved in *in situ* hybridization. This is clearly not enough grains to be obvious on visual inspection of one cell but should be quite clear if data are pooled from a large number of cells.

The advent of recombinant clones derived from the chromosome rather than from mRNA enables fragments of DNA, around 5 kb long, specific to the structural gene but containing intervening and adjacent sequences, to be used as a hybridization probe and therefore allows structural genes also to be detected by this method. We have used such a fragment, 4·4 kb long, to localize the β globin gene to the short arm of chromosome 11 close to the centromere, in agreement with results of chromosome sorting experiments (Lebo *et al.* 1979) and somatic cell hybrids (Jeffreys, Craig & Francke, 1979; Scott, Phillips & Migeon, 1979).

Although nick-translated plasmid DNA may be used directly for hybridization and will in fact result in an enhancement of signal due to the formation of cross-linked molecules (Malcolm *et al.* 1977), we decided to use a complementary RNA transcribed from the plasmid DNA template by *E. Coli* DNA-dependent RNA polymerase because the higher specific activity and the lower background produced by mild RNAase treatment make this more suitable for detecting very low levels of grains. We have found in experiments involving ribosomal and highly repeated sequences that no differences are found when native or heat-denatured DNA is used as the template and therefore there is no specificity of transcription. This is probably because the *E. Coli* RNA polymerase used has only a partial content of σ sub-unit. We have also shown directly that the whole length of the inserted sequence is transcribed under these conditions by hybridization of α^{32}P-labelled cRNA to filters containing restricted plasmid DNA (data not

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.