Volume 10 Number 21 1982 Nucleic Acids Research

Gene amplification in methotrexate-resistant mouse cells. IV. Different DNA sequences are amplified in different resistant lines

Ruggero Caizzi* and Christopher J.Bostock

Medical Research Council, Mammalian Genome Unit, King's Buildings, West Mains Road, Edinburgh EH9 3JT, UK

Received 26 August 1982, Accepted 21 September 1982

ABSTRACT

DNA was purified from double minutes isolated from MTX-resistant EL4/8 mouse lymphoma cells, digested to completion with Bam H1 restriction endonuclease and cloned in λ-1059. The properties of the library suggest that the DNA from which it was made was not detectably contaminated with non-dm chromosome material, and that the library is essentially complete for sequences contained in Bam H1 restriction fragments between 9 and 19 kb. The inserts of some selected λ-recombinants were subcloned in pBR328 or pAT153 to separate sequences of differing repetition frequency. Clones representative of different classes of sequences were used as probes to Southern transfers of Bam H1 digested total nuclear DNAs of various MTX-resistant cell lines. The results clearly show that the amplified unit of each cell line has a unique structure, and that different amplified units differ widely in their sequence composition.

INTRODUCTION

Many studies have shown that the gene for dihydrofolate reductase (dhfr) is amplified in mammalian cells that have been selected *in vitro* for resistance to the antifolate drug, Methotrexate (MTX) (1-7). At the cytological level the acquisition of MTX-resistance is often accompanied by the presence of multiple, free small acentric chromosome structures, called double minutes (dm) (4) or abnormally staining regions called homogeneously staining regions (hsr) which are linked to or integrated into normal chromosomes (8).

The correlation between extra dhfr genes and these abnormal cytologic entities suggests that they are the chromosomal sites of the amplified copies of the dhfr genes. This idea is strengthened by the observation that dhfr gene sequences can be localised to hsr by *in situ* hybridisation (3) and that they are enriched in DNA extracted from dm purified by sucrose gradient centrifugation (4,9).

The size of the normal chromosomal dhfr gene in the mouse is estimated to be about 31 kb (10). This is also the size of the majority of amplified

© IRL Press Limited, Oxford, England.

0305-1048/82/1021-6597$ 2.00/0

dhfr gene copies, although variants and rearranged forms have been found (7,10). By contrast, the average amount of DNA that is amplified for each extra dhfr gene copy has been variously estimated at between 135 and 3000 kb (3,10-13) depending on the particular MTX-resistant cell type examined.

An indication of the size and complexity of the amplified unit has been shown by agarose gel electrophoresis of restriction endonuclease digested DNAs isolated from highly MTX-resistant cells (11,12). The complex patterns of bands in ethidium bromide stained gels, representing amplified DNA sequences, were different in each mouse cell line and were shown to be characteristic for each independently selected MTX-resistant cell line (9). The uniqueness of each pattern and the multiplicity of different patterns showed that the unit of DNA which contains a dhfr gene and becomes amplified, must be formed at a very early stage in the development of resistance by recombination between pieces of DNA from different parts of the genome, or by multiple rearrangements of the sequences which flank the dhfr gene.

As part of our continuing studies on the structure of the amplified unit in MTX-resistant mouse cells we have cloned DNA isolated from purified dm of EL4/8 MTX-resistant mouse lymphoma cell line. Some of these cloned fragments have been used as probes to investigate their amplified status in other MTX-resistant cells. The results confirm the interpretation that there are very large differences in the DNA sequence composition of amplified structures in different, independently selected MTX-resistant cell lines and suggest new levels of complexity in the amplification process.

In this paper we discuss both unique and repeated sequences present in the wild-type genome that have undergone amplification. Since amplification itself results in a sequence becoming repeated, some confusion in terms can result. We reserve the terms "single-copy sequence" and "repeated sequence" to describe the status of a particular sequence before amplification, i.e. in the wild-type genome. Thus, after amplification a cell will contain both amplified single-copy sequences and amplified repeated sequences.

MATERIALS AND METHODS

(a) Cells and Cell Culture

All cells were grown in RPMI 1640 medium (Flow Laboratories) supplemented with 10 per cent (v/v) heat inactivated foetal calf serum; 50 units/ml penicillin; 50 mg/ml streptomycin and, where appropriate, Methotrexate (a gift from Lederle Laboratories). The origin of MTX-

resistant lines of EL4 mouse lymphoma cells, EL4/3, EL4/8, EL4/11 and EL4/12, has been described elsewhere (7). EL4/3 cells were grown in 2×10^{-3}M MTX; the remaining MTX-resistant EL4 lines were grown in 10^{-3}M MTX. PG19T3:$MTX_R^{10^{-4}M}$ (14) and PG19T3:S2 (7) mouse melanoma cells were grown in monolayer culture in medium containing 10^{-4}M and 10^{-3}M MTX respectively. MTX-resistant L5178 mouse leukaemia cells were a gift from Dr. T. Alderson. Originally made resistant to 1.1×10^{-3}M MTX by Courtenay and Robins (15), they were further selected for resistance to 4.4×10^{-3}M MTX by T. Alderson. LtAp20/EL4/8 and LtAp20/EL4/11 are two lines originally selected in 3×10^{-8}M MTX after transfection of LtAp20 cells (a TK^-, $APRT^-$ L cell kindly provided by Dr. F. Graham) with chromosomes isolated from EL4/8 or EL4/11 cells, respectively. Subsequently they were selected for increased MTX-resistance. The DNA used in these experiments was prepared from cells growing at 3×10^{-4}M (LtAp20/EL4/8) and 1×10^{-4}M (LtAp20/EL4/11). Details of the derivation and characterisation of these cells will be provided elsewhere (Bostock and Clark; in preparation).

(b) Phage preparation and bacterial culture

λ-1059 phage were grown in liquid lysate on Q358 bacteria in L-broth supplemented with 10mM $MgSO_4$ (16) and purified by CsCl density gradient centrifugation. To get recombinant phage, Bam H1 digested dm DNA was ligated with Bam H1 digested λ-1059 DNA in a ratio of 1 : 7 using T4 ligase. This DNA was then packaged *in vitro* using a modified protocol of Scalenghe *et al*. (17). Recombinant phage were selected on Q359 bacteria, a host in which only recombinant phage can grow (16).

E. coli HB101 carrying the plasmid pBR328 or pAT153 were grown in L broth containing 25 μg/ml ampicillin. The subclones pdm45-7.3 and pdm45-5.3; pdm87-8.2 and pdm87-5.1 were constructed from λ-dm45 and λ-dm87, respectively, by digesting the latter cloned DNA with Bam H1 and ligating to Bam H1 digested pAT153 vector DNA. Ligated DNA was used to transform HB101 cells (18) and ampicillin-resistant, tetracycline-sensitive cells were selected. The subcloning of the 3'-end of the dhfr gene into pBR328 (pdm31-4.9) was performed in a similar manner by C. Tyler-Smith. The plasmid pDHFR11 which carries the dhfr cDNA was a gift of Dr. R.T. Schimke.

(c) Purification of double minutes

Suspension cultures of EL4/8 cells in late exponential phase of growth were diluted with an equal volume of fresh prewarmed medium 24 hr. prior to the addition of colchicine to a final concentration of 2×10^{-6}M. After

16 hr. exposure to colchicine the cells were harvested by centrifugation at 400 x g for 5 mins., and chromosomes prepared by the polyanionic buffer method described elsewhere (19,20). Suspensions of lysed cells containing free nuclei, chromosomes and dm were centrifuged at 800 x g for 10 mins. at $0^{o}C$ to remove the bulk of nuclei and chromosomes. The supernatant was removed and passed through a 3μ pore size, and secondly through a 1μ pore size, Nucleopore filter. The small dm readily pass through these filters whereas any contaminating chromosomes are retained by the filters. The filtrate was centrifuged at 9,000 x g to sediment the dm.

(d) Preparation of DNA

DNA was prepared from whole cells or purified nuclei as described by Tyler-Smith and Bostock (9). DNA was prepared from recombinant phage using a mini-lysate procedure (Cooke, personal communication). Briefly, a single plaque was picked out and used to infect 50 μl of saturated Q358 bacteria. The phage were then amplified in 5 ml L broth overnight at $37^{o}C$. DNA was prepared from phage concentrated by polyethylene glycol 6000 precipitation using phenol, phenol-chloroform and chloroform extraction. The DNA was ethanol precipitated and redissolved in 40 μl 10mM Tris; 1 mM EDTA, pH = 8.4. Usually 4 to 10 μl were used for agarose gel electrophoresis. The procedure of Ish-Horowicz and Burke (21) was used for the preparation of DNA from bacterial mini-cultures.

(e) Restriction enzyme digestion and DNA electrophoresis

Eco R1 was prepared in this laboratory by Mrs. Barbara Smith. XhoI and HindIII were purchased from New England Biolabs. Bam H1 was purchased from Boehringer Mannheim. Enzymes were used in a 2 to 5-fold excess over that expected to be necessary to completely digest DNA under standard conditions. The digestion buffers were those recommended by the suppliers. For gel electrophoresis, digested DNA was precipitated with ethanol, dried and redissolved in distilled water. Aliquots were added to electrophoresis buffer (see below) containing 3 per cent (w/v) Ficoll and a trace of Orange G, and loaded into the slots of vertical 0.8% or 1.0% agarose gels. Electrophoresis buffer was 26 mM Na_2HPO_4, 3.3 mM NaH_2PO_4, 1 mM EDTA and gels were run at a voltage gradient of 1 to 2 V/cm. Size markers were either Eco R1 digested λ-c1857 or a mixture of XhoI and HindIII digests of λ-c1857.

(f) Labelling of DNA and hybridisation

DNAs were labelled to a specific activity of about 10^{8} disintegrations/min/μg by nick-translation (22) and used as probes to electrophoretically

separated DNA fragments after transfer to nitrocellulose filters (23) or to nitrocellulose filters containing replicas of λ-1059 recombinant plaques (24). The subclones in pAT153 or pBR328 were selected by sizing the insert DNA of the plasmid and comparing the size with that of the corresponding fragment in a Bam H1 digest of the λ-recombinant DNA.

(g) Screening for λ-recombinants containing DNA inserts of varying repetition frequency

We used an approach which is similar in concept, but which is experimentally simpler, to that described by Brison et al. (25). 1 μg of total nuclear DNA from EL4/8 cells was labelled by nick-translation (22) and sheared by ten passes through a 25G hypodermic needle. Unincorporated ^{32}P-labelled deoxynucleotide triphosphates were removed by G-50 (medium) chromatography. Fractions containing incorporated radioactivity were pooled, made up to 0.15 M sodium acetate, 10 μg/ml yeast transfer RNA and precipitated by the addition of 2 volumes of absolute ethanol. After leaving at -70°C for 30 mins., the precipitate was collected by centrifugation, washed once with absolute ethanol and dried. The precipitate was redissolved in 264 μl distilled water and then made up to 0.12 M sodium phosphate (pH = 7.0) by the addition of 36 μl of 1 M sodium phosphate buffer. The tube was sealed, heated to 100°C for 5 mins. and incubated at 65°C for three hours, i.e. to a C_ot value of about 10^{-1}. 0.1 ml of hydroxylapatite (HAP) was added to adsorb reassociated molecules. After centrifugation the supernatant was removed and treated with a further aliquot of HAP. This treatment removed approximately 40 per cent of the incorporated radioactivity. The resulting non-reassociated single-stranded ^{32}P-labelled DNA was divided into two equal portions, each containing about 0.25 μg ^{32}P-labelled probe, and 250 μg of sonicated unlabelled total DNA from EL4 wild-type was added to one half. Both tubes were sealed, heated to 100°C for 5 mins. and used as hybridisation probes to replica nitrocellulose filters containing plaques of λ-recombinants with dm DNA inserts.

The initial reassociation has the effect of removing the bulk of highly repeated sequences in the mouse genome, but will leave single-stranded any sequence repeated 10,000 times or less. The amplified unit, which contains the dhfr gene, is present in about 1,000 copies per EL4/8 haploid cell and, thus, the concentration of these amplified sequences relative to non-amplified sequences in the same cell will not be markedly altered. The addition of 1,000-fold excess of "competing" unlabelled wild-type DNA has the effect of "diluting" the specific activity of all non-

amplified sequences by 1,000-fold or greater, while only reducing the specific activity of amplified single-copy sequences by a factor of two. Amplified sequences that are repeated in wild-type cells will be "competed" to varying extents depending on the repetition frequency in non-amplified DNA and their relative abundance in the amplified unit. A comparison of the density of autoradiographic grains resulting from hybridisation to the plaques with or without competition allows the identification of λ-recombinants carrying inserts of only amplified single-copy sequences and those carrying either mainly low-copy number amplified repeated sequences or those with only a small proportion of amplified repeated sequence DNA. This method is referred to as the "plus/minus competitor" method.

RESULTS

Purity of double minutes

Microscopic examination of a fixed and stained preparation of dm showed essentially no contamination with normal chromosomal material. In a field containing hundreds of dm, only rarely was a small mouse chromosome or a fragment of a normal chromosome seen. No attempt was made to quantify by cytological means the relative contributions that dm and normal chromosomes would make to the final DNA preparation. Instead, the purity of the dm preparation was estimated by measuring the enrichment for dhfr genes in dm DNA with respect to the concentration of dhfr genes in total nuclear DNA from EL4/8 cells. Figure 1 shows such a comparison. Although the hybridisation signals with pDHFR11 are the same in tracks 5 (EL4/8 nuclear DNA) and 6 (dm DNA), the amount of dm DNA loaded (track 2) was approximately 35 ng; one seventh the amount of total nuclear DNA (250 ng) loaded in track 1. Thus, purification of dm resulted in a seven-fold enrichment for dhfr genes, which is similar to the maximim possible enrichment (9).

Characteristics of the λ-1059 library of Bam H1 fragments of dm DNA

Because of the purity of the dm, we made the working assumption that essentially all λ-recombinants containing DNA inserts of mouse origin would be derived from dm DNA. That such an assumption is, to a first approximation, correct is shown both by the plus/minus competitor screening (see below) and by the fact that all recombinants used as probes against Southern transfers of resistant and wild-type DNAs showed patterns of amplification in EL4/8 DNA and often other MTX-resistant cell DNAs.

120 recombinants were picked at random from the first plating of the

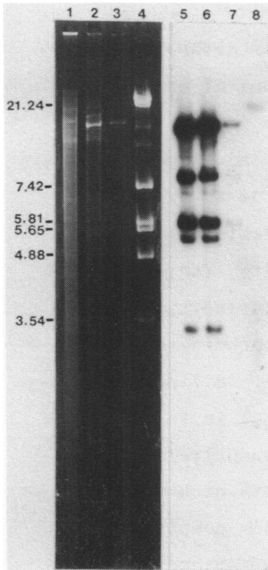


Figure 1 Enrichment of dhfr genes in purified dm DNA. DNA was purified from isolated EL4/8 nuclei (tracks 1 and 5), purified EL4/8 dm (tracks 2 and 6) and the 9000 x g supernatant from EL4/8 dm (tracks 3 and 7), digested with Eco R1 restriction endonuclease and the resulting fragments separated by electrophoresis in 1.0 per cent agarose gels. Tracks 1 - 4 show ethidium bromide stained DNA fragments. Tracks 5 - 8 show an autoradiograph of the same samples following Southern transfer and hybridisation with ^{32}P-nick translated pDHFR 11. In track 4 are fragments of an Eco R1 digest of λ-c1857 DNA used as size markers. The sizes in kb of the λ fragments are shown on the left.

library. These were grown in mini-culture and, after Bam H1 digestion, their DNA was analysed by gel electrophoresis. The ability of the inserts to hybridise either nick-translated pDHFR11, total EL4/8 DNA or total EL4 wild-type DNA showed firstly, whether they contained any dhfr coding sequences, secondly, whether the inserts were mouse DNA and, thirdly, what their relative content of repeated or single-copy sequences might be. The sizes of 130 Bam H1 fragments derived from inserts were estimated relative to the sizes of the left and right hand arms of λ-1059 and known fragment sizes of XhoI, HindIII and Eco R1 digested λ-c1857 DNA. 60 of the Bam H1 fragments cloned as single inserts were between 9 and 19 kb; the apparent optimum size range for cloning in λ-1059 in our hands. Although λ-1059 is reported to accommodate single DNA fragments of between 6 and 24 kb in length (16), we never found single DNA inserts of less than 9 kb or greater than 19 kb. The remaining 70 were smaller than 9 kb and ligated to other fragments to form inserts of the necessary size to clone in λ-1059.

From these initial 120 λ-recombinants, 6 were chosen that contained representatives of the different types of sequence of interest. Those recombinants containing two Bam H1 restriction fragments of dm DNA ligated together to form the insert were subcloned into pAT153 to separate the two Bam H1 fragments. The 4.9 kb fragment in λ-dm 31, which is the 3' end of the normal dhfr gene, was subcloned into pBR328. The various λ clones and

plasmid clones used in this study are summarised in Table 1.

The library was screened by the plus/minus competitor method (see Materials and Methods). Figure 2 shows equivalent sections of each of the replica filters; this area covers approximately one third of the total surface of the 20 x 20 cm plate. In comparing Figure 2A (no competitor) with Figure 2B (plus competitor) the first impression is of an overall reduction in the level of hybridisation upon adding competitor. While the level of hybridisation is drastically reduced in many spots, (an example of one of these is circled) a closer inspection shows that intensities of a number of spots are approximately the same in the two autoradiographs (closed arrows) or differ by a small factor (open arrows). 4.7 per cent of all the plaques give about the same hybridisation signal in the presence or absence of competitor. These plaques contain the presumptive single-copy sequences which have been amplified as part of the DNA of a dm.

We investigated further the validity of the plus/minus competitor method of screening, and characterised the λ-recombinants tentatively identified as having single-copy, low repeat, or high repeat inserts. To do this, nine presumptive single-copy, six presumptive low repeat and one presumptive high repeat recombinants were picked and grown in miniculture without further plaque purification. DNAs purified from these recombinants, together with DNA from λ-dm31, λ-dm43, λ-dm45 and λ-dm87 were digested with

TABLE 1

SUMMARY OF CLONED SEQUENCES

λ-recombinant	Size of insert (kb)	Sub-clone	Sequence characteristic
λ-dm31	6.1		high repeat
	4.9	pdm31-4.9	3' end of dhfr gene
λ-dm43	ca. 14.0		low repeat
λ-dm45	7.3	pdm45-7.3	high repeat
	5.3	pdm45-5.3	single-copy
λ-dm58	11.0		low repeat
λ-dm87	8.2	pdm87-8.2	single-copy
	5.1	pdm87-5.1	high repeat
λ-dm88	ca. 17.0		low repeat
λ-dm3s*	ca. 13.0		single-copy

*Identified on plus/minus competitor screen

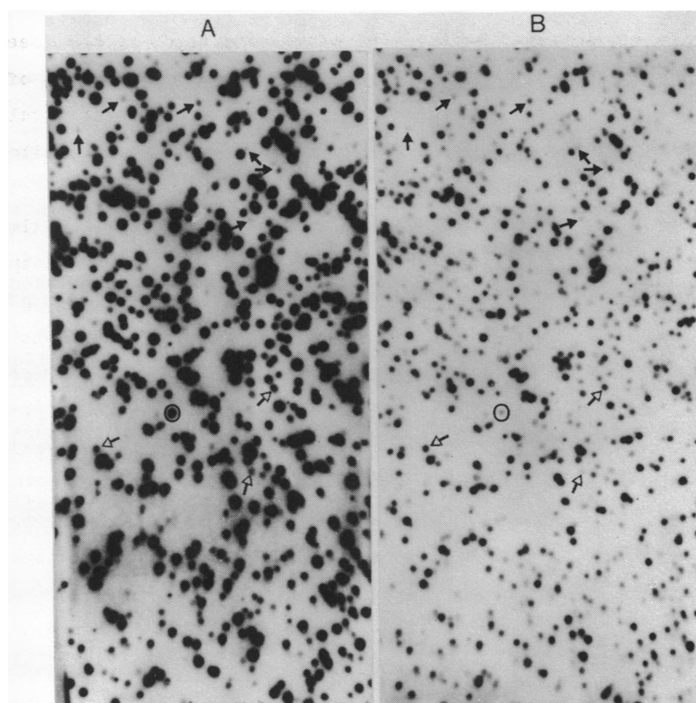


Figure 2 Screening for sequences of different repetition frequency in the library of Bam H1 digested dm DNA cloned in λ-1059.
A and B are portions of replica nitrocellulose filters containing plaques of λ-dm recombinants. The filters were hybridised to ^{32}P-nick translated EL4/8 DNA in the absence (A) or presence (B) of a 1000-fold excess of unlabelled wild-type mouse DNA. Closed arrows indicate presumptive single-copy sequence clones, open arrows indicate presumptive low-repeat sequence clones and the circle indicates a presumptive high-repeat sequence clone.

Bam H1 and the resulting fragments separated by gel electrophoresis. Duplicate gels were run and duplicate nitrocellulose filters prepared by Southern transfer. The transfers were probed by the plus/minus competitor method and the resulting autoradiograms are shown in Figure 3. A number of features are evident.

All nine "single-copy" recombinants (lanes 13 to 21) contain a 13 kb insert which produces the same hybridisation signal irrespective of the presence or absence or competitor DNA. Lanes 13, 17 and 20 contain additional large inserts but these fragments contain repeated sequences, since their hybridisation signal disappears completely in the presence of competitor. The "single-copy" recombinant plaques represented in lanes

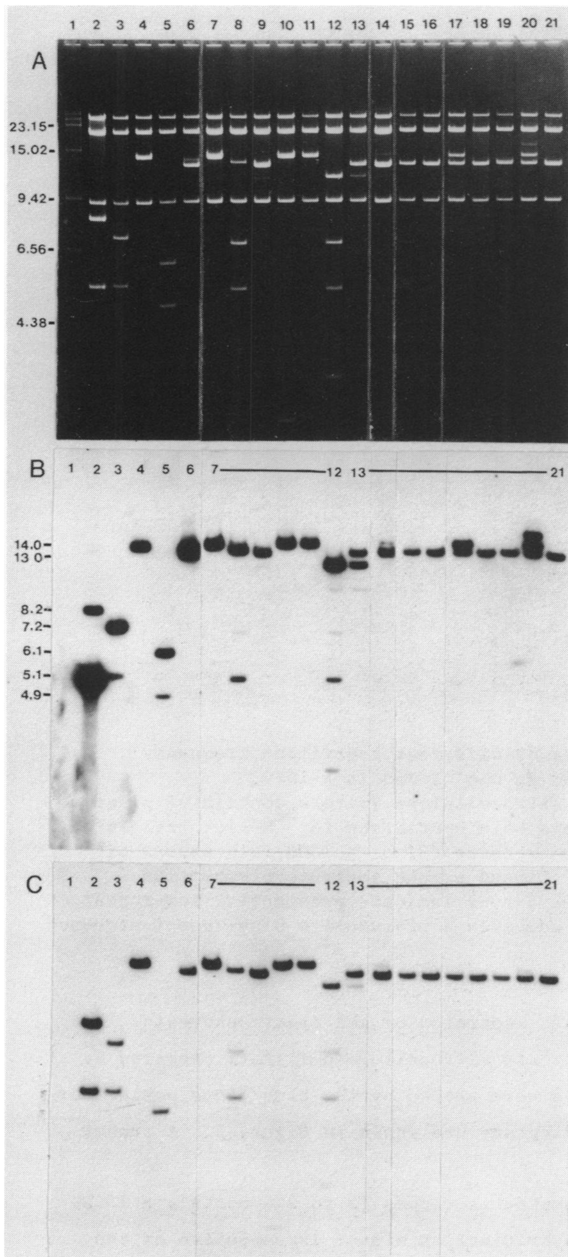


Figure 3 Analysis of cloned dm DNA inserts by the plus/minus competitor method. (A) Ethidium bromide stained 1 per cent agarose gel of Bam H1 digested DNAs. (B) and (C) replica Southern transfers of gels identical to that shown in (A), which have been hybridised to ^{32}P-nick translated EL4/8 DNA in the absence (B) or presence (C) of a 1000-fold excess of unlabelled wild-type mouse DNA. Track 1, a mixture of fragments produced by digestion of λ-c1857 with XhoI or HindIII restriction endonucleases. The sizes in kb of the fragments are shown on the left of (A). Track 2, λ-dm87. Track 3, λ-dm45. Track 4, λ-dm43. Track 5, λ-dm31. Track 6, presumptive high-repeat sequence clone. Tracks 7 - 12, presumptive low-repeat sequence clones. Tracks 13 - 21, presumptive single-copy sequence clones. The sizes in kb of the major Bam H1 fragments of dm origin are indicated on the left of (B).

13, 17 and 20 were lying adjacent to high repeat sequence plaques and are thus presumably composed of a mixture of two recombinants (lanes 13 and 17) or three recombinants (lane 20). The recombinant shown in lane 19

is λ-dm3s, referred to in Table 1 and Figure 4.

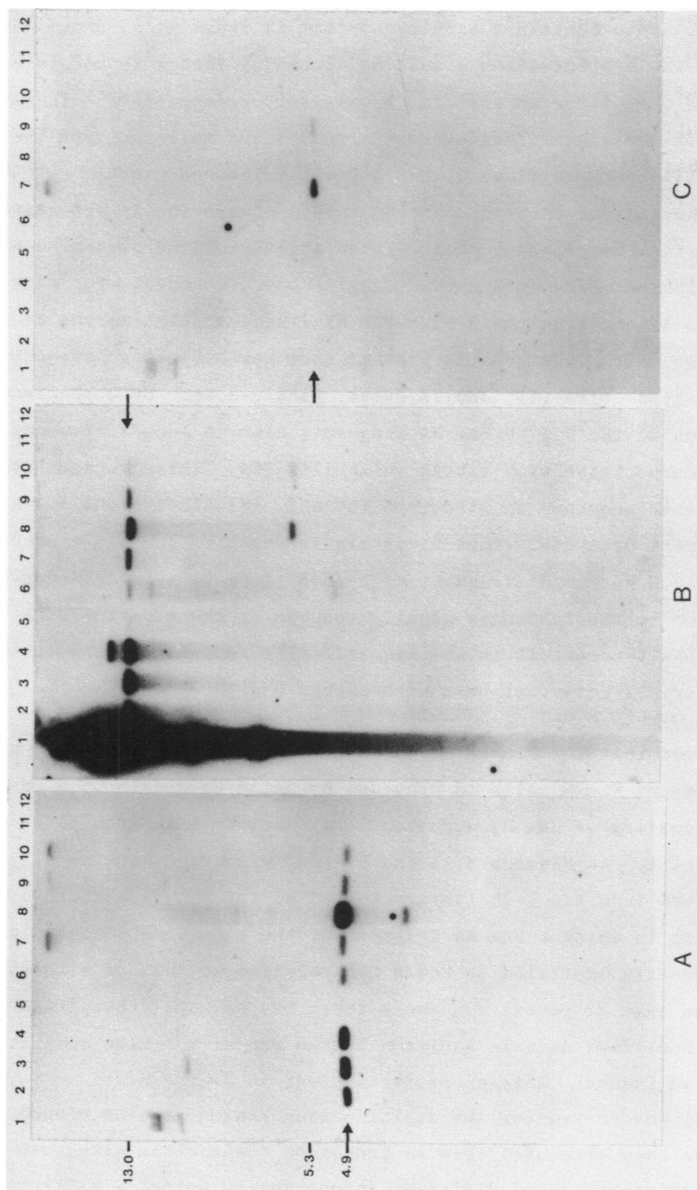
The six "low-repeat" recombinants are distinct from each other, having different sizes of insert, although the recombinants in lanes 7 and 12 would appear to contain a single-copy Bam H1 fragment of about 7.0 kb in common. The hybridisation signal of the major insert is moderately reduced with competitor in all six "low-repeat" recombinants. In contrast, the insert of the single "high-repeat" recombinant selected from the plus/minus competitor screen (lane 6) has a greatly reduced signal.

The behaviour of the four λ-recombinants of previously determined composition in lanes 2 and 5 confirms the ability of the plus/minus method to distinguish between repeated and single-copy sequences. For example, λ-dm31 (lane 5) contains the 4.9 kb Bam H1 fragment which is the 3' end of the dhfr gene. The dhfr gene is presnet once per haploid genome equivalent in EL4 wild-type cells (7), and is, thus, single-copy. By comparison, hybridisation to the 6.1 kb Bam H1 fragment, also in λ-dm31, is completely removed by competition with excess unlabelled DNA. This fragment is a highly repeated sequence in wild-type EL4 DNA. Similarly, the 5.1 kb Bam H1 fragment of λ-dm87 (lane 2) is highly repeated in wild-type DNA, whereas the 5.3 kb Bam H1 fragment of λ-dm45 (lane 3) is single-copy. Hybridisation to the former is greatly reduced by the addition of competitor DNA, whereas it is not significantly reduced for the latter.

Probing other MTX-resistant DNAs with cloned DNA sequences of EL4/8 dm

The proof that the cloned sequences are part of the amplified unit present in dm of EL4/8 cells is provided by using them as probes to Southern transfers of Bam H1 digested total nuclear DNAs from EL4/8 and wild-type cells. As Figures 4, 5 and 6 show, without exception all clones derived from the λ-dm library tested so far show a pattern of hybridisation in which a Bam H1 fragment of the same size as the cloned insert is greatly amplified in EL4/8 DNA relative to that of wild-type DNA. In the case of repeat sequences there may be many other fragments within the amplified unit in addition to the one with a size characteristic of the cloned insert. This property is clear in Figure 6.

In addition to testing the amplification status of each cloned sequence, we have also used them as probes to compare the structure of amplified units in several different independently selected MTX-resistant cell lines.


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
13.0
5.3
4.9

(a) Amplified single-copy sequences

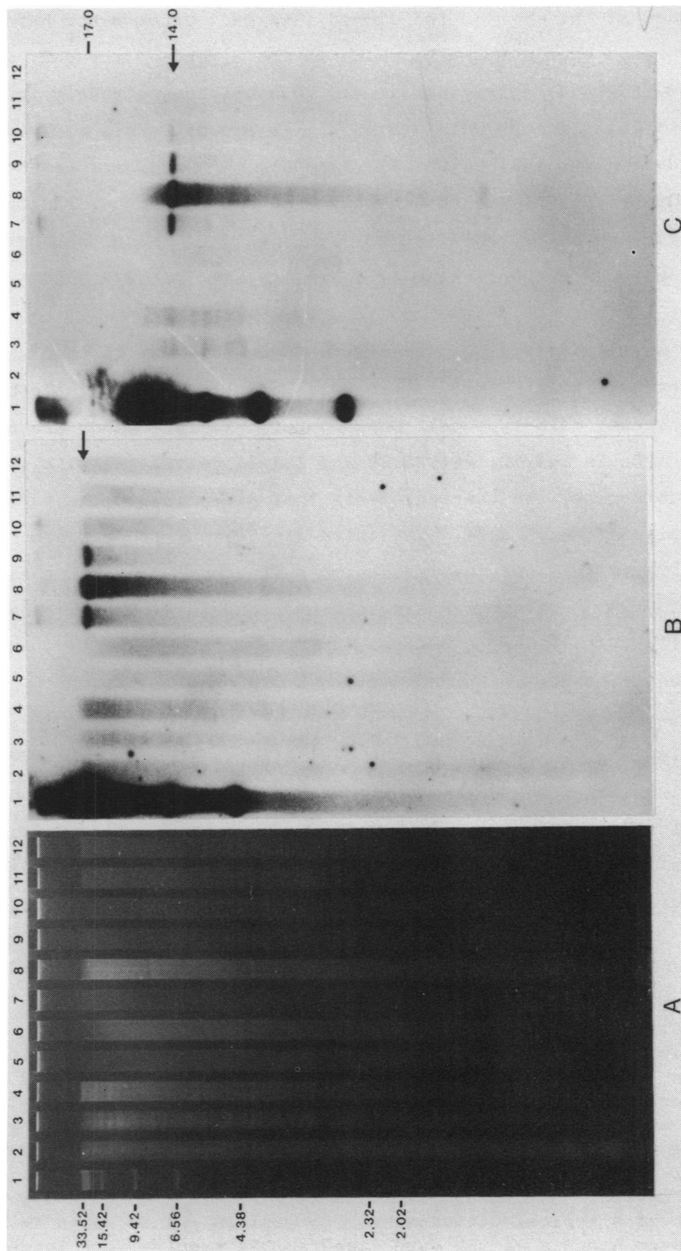
Hybridisation of amplified single-copy probes reveals a major prominent band at the size of the cloned inserts. Other minor bands may also be present. When pdm31-4.9, which is the 3' end of the dhfr gene, is the probe (Fig. 4A) a fragment at 4.9 kb hybridises strongly in all MTX-resistant cells except PG19T3:MTX_R 10^{-4}M (lane 5). This cell has ten-fold fewer dhfr genes that PG19T3:S2 (lane 6). No bands are detected in wild-type DNAs (lanes 11 and 12) at this exposure of the autoradiograph. Characteristic bands, representing rearrangements at the 3' end of some amplified copies of the dhfr gene (7), are visible in EL4/3, EL4/8 and EL4/11 DNAs.

A very similar overall pattern is found when λ-dm3s is used as a probe (Figure 4B). All MTX-resistant cell DNAs have a detectable band at 13 kb, whereas, wild-type cell DNAs do not. Variant bands are also found, this time in EL4/12, PG19T3:S2 and EL4/11 cells. As with the 3' end of the dhfr gene, the rearrangements that give rise to the variant bands are unique to each cell. The third single-copy probe shown, pdm45-5.3 (Figure 4C), only hybridises to a fragment of the same size in EL4/8 DNA. Thus, this particular single-copy sequence is amplified only in EL4/8 cells.

(b) Amplified low-repeat sequences

The low-repeat sequences shown in Figure 5 both hybridise predominantly to a single band of the same size as that of the insert fragment. λ-dm88 (Figure 5B) also produces a distinctive background smear in all MTX-resistant cells, but this cannot be due to amplified sequences as it also is detected to the same extent in wild-type cells. λ-dm43 (Figure 5C) produces a light background smear detectable also in

Figure 4 Hybridisation of cloned single-copy sequences from dm to Bam H1 digested DNAs of different MTX-resistant cell lines. The Bam H1 fragments were separated by electrophoresis in 1 per cent agarose gels, transferred to nitrocellulose filters and hybridised with ^{32}P-nick translated pdm31-4.9 (A), λ-dm3s (B) and pdm45-5.3 (C). The DNAs in the tracks are as follows: 1, mixture of XhoI and HindIII generated fragments of λ-c1857; 2, L5187Y; 3, EL4/3; 4, EL4/12; 5, PG19T3:MTX_R 10^{-4}M; 6, PG19T3:S2; 7, EL4/8; 8, EL4/11; 9, LtAp20/EL4/8 transformant; 10, LtAp20/EL4/11 transformant; 11, wild-type PG19T3; 12, wild-type EL4. A photograph of a typical ethidium bromide stained gel is shown in Figure 5A - the same amounts of the same digested DNA samples were loaded in all the gels shown in Figures 4, 5 and 6. The arrows and numbers indicate hybridisation to genomic fragments of the same size (in kb) as the cloned inserts.



1 2 3 4 5 6 7 8 9 10 11 12
33.52-
15.42-
9.42-
6.56-
4.38-
2.32-
2.02-
A
B
C
-17.0
14.0

wild-type cells, but with this probe faint clear bands can be identified in EL4/3, EL4/12 and EL4/11 cells. Notice that they are not all of the same sizes in these three cells. Another low-repeat probe, λ-dm58 (not shown), hybridises strongly to a few bands in several MTX-resistant DNAs, but the pattern of bands differs markedly between the different cells in a manner similar to that shown for the high-repeat clones pdm87-5.1 and pdm45-7.3 in Figure 6. Both λ-dm88 and λ-dm43 contain inserts classified as low repeats because of the ability of these probes to hybridise to a number of copies of a sequence which is repeated and dispersed throughout the mouse genome. In both EL4/8 and EL4/11 cells, only one of these dispersed repeats, contained within a 17 kb (λ-dm88) or a 14 kb (λ-dm43) Bam H1 fragment was amplified. These fragments are not part of the amplified units of other MTX-resistant cell lines.

(c) Amplification of high-repeat sequences

Figure 6 shows the hybridisation of two cloned Bam H1 fragments from EL4/8 dm DNA; pdm45-7.3 (Figure 6B) and pdm87-5.1 (Figure 6A). Because of the similarities of the patterns, it is clear that they contain at least part of their sequences in common. They both hybridise strongly to a 4.1 Bam H1 fragment, which is also repeated in wild-type DNAs, so they must both contain part of a sequence complementary to this sequence.

There are several thousand copies of the 4.1 kb Bam H1 fragment in total wild-type mouse DNA. Thus, if one or two copies of this fragment were incorporated into an amplified unit, present some 1,000 times per cell in MTX-resistant cells, it would be difficult to detect. Much of the variation in intensity of hybridisation signal to the 4.1 kb Bam H1 fragment is due to variation in the amount of DNA loaded onto the gel (see the ethidium bromide stained photograph in Figure 5A). However, it is clear that in EL4/8 cells (from which the dm were isolated) few copies of the 4.1 kb fragment have been amplified.

Figure 5 Hybridisation of cloned low-repeat sequences from dm to Bam H1 digested DNAs of different MTX-resistant cell lines. The loading of MTX-resistant DNAs in gel tracks is as described in the legend to Figure 4. (A) is a photograph of an ethidium bromide stained gel to show the variation in the amount of DNA loaded in each track. The differences between tracks are the same for all the gels represented by Southern transfers in Figures 4, 5 and 6. (B) Southern transfer hybridised with ^{32}P-nick translated λ-dm88. (C) Southern transfer hybridised with ^{32}P-nick translated λdm43. The numbers on the left are the sizes in kb of the λ-c1857 marker fragments. The numbers on the right are the sizes in kb of the genomic fragments of the same size as the cloned inserts with which they hybridise.

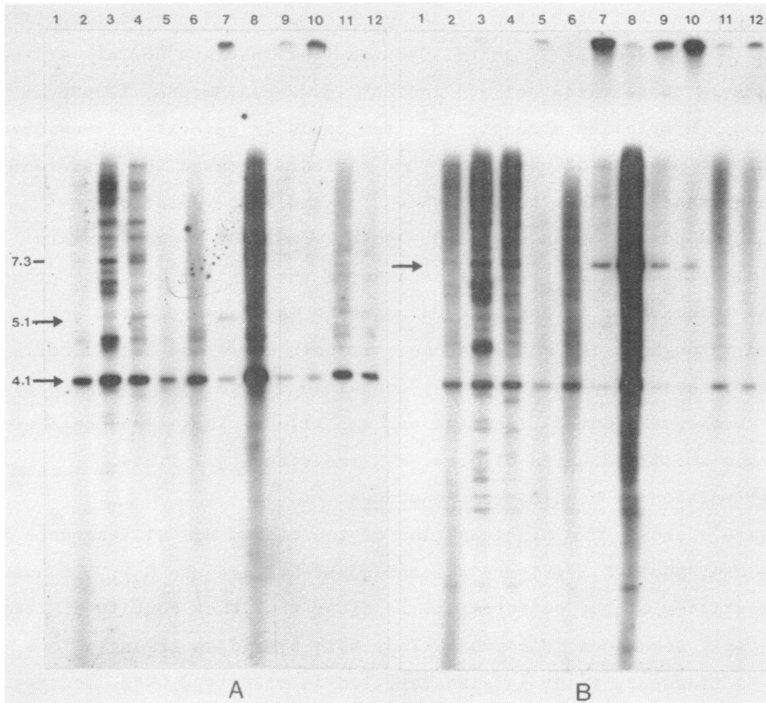


Figure 6 Hybridisation of cloned high-repeat sequences from dm to Bam H1 digested DNAs of different MTX-resistant cell lines. The loading of MTX-resistant DNAs in gel tracks is as described in the legend to Figure 4. Southern transfers have been hybridised with ^{32}P-nick translated pdm87-5.1 (A) and pdm45-7.3 (B). The Bam H1 generated genomic fragments in EL4/8 equivalent to these inserts are arrowed with the sizes indicated in kb on the left. Also arrowed, at a size of 4.1 kb, is the wild-type mouse repeated sequence which can be seen in all mouse DNA tracks of the gel stained with ethidium bromide in Figure 5A.

A surprising feature of the hybridisation patternsof pdm45-7.3 and pdm87-5.1 is the overall similarity of fragments detected in other MTX-resistant and wild-type cells, but apparent lack of homology between the 7.3 kb and 5.1 kb fragments in EL4/8 cells. pdm45-7.3 clearly detects a 7.3 kb fragment in EL4/8 DNA (Figure 6B, lane 7), but barely hybridises to the 5.1 kb fragment, whereas, pdm87-5.1 has virtually no homology with the 7.3 kb fragment (Figure 4A, lane 7) but does hybridise to an amplified 5.1 kb fragment. Since both hybridise to comparable extents to the 4.1 kb fragment in all DNAs and several other bands in EL4/3 and EL4/12 DNAs, it would seem that pdm45-7.3 and pdm87-5.1 contain different parts of the 4.1 kb MIF sequence.

The different patterns of hybridisation of these two cloned repeated sequences clearly demonstrate the large differences that exist in the structures of amplified sequences in different MTX-resistant lines. With these probes, DNAs from MTX-resistant L5178Y and PG19T3 cells are indistinguishable from wild-type mouse cells. EL4/8 cells have amplified perhaps 5 Bam H1 fragments with homology to these probes. EL4/3 and EL4/12 have similar, but distinguishable patterns composed of about 20 different amplified Bam H1 fragments. Lastly, EL4/11 has a large and complex composition of Bam H1 fragments which are homologous to these probes.

DISCUSSION

The library of dm DNA in λ-1059 was prepared from DNA digested to completion with Bam H1 restriction enzyme. From high resolution agarose gels of total EL4/8 DNA digested to completion with Bam H1 (see Ref. 11) we can estimate that the amplified unit contains approximately 20 different Bam H1 fragments between 9 and 19 kb in size. There are another three above the size that we were able to clone in λ-1059 and about 50 fragments smaller than 9 kb, i.e. below the optimum cloning size. The library must therefore be incomplete, since approximately 60 kb will be absent due to the three large Bam H1 fragments, and many of the smaller fragments would be either underrepresented or absent. These small fragments would be present only if joined together by a chance ligation event to make fragments of a clonable size. We estimate, therefore, that any single Bam H1 fragment of between 9 and 19 kb in size would be present at about twice the relative concentration that it occurs in pure dm DNA.

In the plus/minus competitor screen we identified 37 out of 794 plaques (shown in Figure 2) as being potentially single-copy. Nine of these, picked at random, were found to be the same single-copy sequence, of which λ-dm3s is just one example. It is likely that most of the 37 plaques also contain the same single-copy sequence. At what frequency would we expect to find a particular 13 kb sequence in the λ library? It is difficult to estimate accurately the size of the amplified unit in EL4/8 cells, but summing the sizes of all bands visible in ethidium bromide stained gels of Bam H1 or Eco R1 digested EL4/8 total DNA gives a size of about 600 kb. This is almost certainly an underestimate because of the difficulties in visualising fragments of low molecular weight and in allowing for fragments present in different molar ratios. A single 13 kb segment would represent some 2.25 per cent of a 600 kb unit, so, in a complete

library we might expect about 2.25 per cent of the recombinant plaques to contain this sequence. We have already noted that the library is not complete and that a 13 kb fragment would be expected to be present at roughly twice the frequency it occurs in the complete amplified unit. The observed frequency of 4.7 per cent is therefore close to our estimate of an expected frequency of about 4.5 per cent. This can be taken as another indication that the DNA isolated as dm was essentially free of contaminating non-dm DNA. The presence of non-dm DNA would have "diluted" amplified sequences and would have resulted in a decreased incidence of clones carrying the 13 kb single-copy insert.

The results from probing Southern transfers of various MTX-resistant DNAs with cloned probes derived from dm of EL4/8 show how very different are the amplified units in these cells. This confirms and extends our earlier observations on ethidium bromide stained gels of total nuclear DNAs (11). In this paper we have shown a few examples of the variations that can be observed. These are:

(1) Wild-type single-copy sequences which become amplified in only one MTX-resistant cell (pdm45-5.3).

(2) Wild-type single-copy sequences which become amplified in all MTX-resistant cells, but with sub-populations of amplified units in particular MTX-resistant cells containing variant forms (pdm31-4.9 and λ-dm3s). λ-dm3s is presumably located close to the dhfr gene in the mouse genome.

(3) Single copies of low-repeat sequences in wild-type DNA which become amplified in only two of the seven MTX-resistant cells studied here (λ-dm43 and λ-dm88).

(4) Different copies of a high-repeat sequence in wild-type DNA which become amplified in different cells (λ-dm58, pdm45-7.3 and pdm87-5.1).

Although a variety of MTX-resistant cells have been characterised in considerable detail, few studies have compared the structure of the amplified unit in different MTX-resistant cell lines. In one such study, Schilling et al. (10) isolated a number of overlapping recombinant DNA clones which defined a region of about 150 kb around the dhfr gene in the amplified unit of MTX-resistant S-180 cells. Using sub-cloned single-copy sequences located in the flanking regions of the S-180 amplified unit, Schilling et al. compared the structures of the amplified units in 3T3-R50 and two lines of L5178Y cells. The stucture of the amplified units is the same in the four cell lines over a region covering about 70 kb in and

around the gene; from approximately 28 kb on the 5' side of the gene to about 15 kb on the 3' side of the gene. Beyond these limits, homology between the units in different cells breaks down completely or sub-populations of variant units are found. Since the size of the amplified unit is estimated to be between 500 and 1,000 kb (10) the finding that sequences diverge so close to the dhfr gene suggests that in S-180, 3T3-R50 and L5178Y cells, as in those described in this report, there are large structural differences between the major portion of the amplified units in each of the cells studied.

Within the library of S-180 cell DNA, two types of variant structures were found, each occurring at one-tenth the frequency of the main structure. One of these variants involves a rearrangement within the middle of the gene and is believed to be a partial gene, the other represents a rearrangement just upstream from the normal start codon. These rearrangements are very similar to those found around the dhfr genes in various MTX-resistant EL4 cell lines (Ref. 7 and Figure 4A). A similar phenomenon probably accounts for the bands of low intensity and differing size detected by the single-copy sequences cloned in λ-dm3s (Figure 4B).

In contrast to the apparent variation between amplified structures formed by different events in different cells is the stability of the structure once it is formed. The structure of dm of MTX-resistant PG19T3 cells is maintained through sequential selection to higher resistance levels and clonal growth. The conversion of dm to stable chromosome forms in EL4/8 cells also conserves the detailed structure of the amplified unit (9). More surprisingly, the entire structure can be maintained throughout transfection of mouse L cells with EL4/8 or EL4/11 chromosomes and during selection of the resulting transformants to high levels of MTX-resistance. This is apparent from the similarities of lanes 7 and 9, or lanes 8 and 10 in Figures 4, 5 and 6. A more detailed analysis of the amplified unit in these MTX-resistant transformants will be presented elsewhere (Bostock and Clark; in preparation).

Before selection for MTX-resistance is applied the dhfr gene is contained within a well conserved "wild-type" chromosomal environment. Once MTX-resistance is established, extra copies of the dhfr gene are contained in an amplified structure which can be conserved through increases or decreases in dhfr gene number, changes in location between dm and hsr, and transformation into L cells with subsequent further amplification. This latter structure we call the "stable amplified unit",

and when contained in this the dhfr gene has a different chromosomal environment from its wild-type location.

For any particular cell line the stable amplified unit contains a constant ratio of normal to rearranged dhfr genes, so the unit must contain more than one copy of the dhfr gene. The ratio of normal to rearranged gene fragments detected in EL4/11 cells (see Figure 4A, track 8) suggests that the amplified unit contains at least five genes with the normal structure for each rearranged copy. On the other hand, EL4/11 cells contain approximately equimolar ratios of the normal and rearranged forms of the 13 kb sequence (represented by λ-dm3s). This suggests that the stable amplified unit is formed in several stages. The first structure to be formed may have included two copies of the dhfr gene and some of its flanking sequences, but during its formation one of the duplicates of the 13 kb sequence may have become rearranged. Subsequent amplification of this "duplicate" structure would maintain the equal ratio of normal to re-arranged forms. One of the dhfr genes may have become rearranged in a subsequent duplication event when the growing unit contained several dhfr genes. At some stage, however, rearrangements must cease to occur at detectable levels and a unit of DNA is formed that is resistant to any further changes in structure.

At least some of the heterogeneity between independently derived amplified units may therefore be generated by the rearrangements that take place between the time that a single dhfr gene is located in a chromosome and the stage at which several amplified copies are stabilised in an amplified unit. Some heterogeneity must also derive from the original choice of DNA to be included in the structures formed during the early stages of amplification. For example, the sequence contained in pdm45-5.3 is only found in EL4/8 cells. However, the choice is not entirely random since independently derived MTX-resistant lines do show some similarities; for example, the sequences that are homologous to pdm87-5.1 in EL4/3 and EL4/12 cells and those that are homologous to λ-dm88 or λ-dm43 in EL4/8 and EL4/11 cells.

In cells selected for a defined resistance it can be shown by a variety of methods that the cytologically identified abnormal chromosomal structures are the sites of localisation of amplified genes. There are, however, a number of spontaneously arising tumours which contain cytologically similar aberrant chromosome forms (e.g. 26) but which have an unknown DNA sequence composition. By analogy with resistant cells we might

suppose that these dm and hsr are also composed of amplified sequences that are perhaps necessary for maintenance of the transformed or tumourigenic phenotype.

In the two naturally occurring tumour systems studied to date, namely mouse Y1-DM adrenocortical tumour cells (27,28) and human colon carcinoid cells (29), structural features of dm, which are similar to those reported here for dm of MTX-resistant cells, have been described. Firstly, cloned DNA sequences from dm are amplified in the cells of origin. Secondly, the few sequences that have been studied are only amplified in cell lines derived from the same starting tumours, but are not amplified in related or unrelated tumours. Thirdly, when in the amplified state, the sequences are contained in a restriction fragment or fragments of different size to those in which they occur in the normal non-amplified state. This suggests that during the formation of dm prior to amplification, multiple re-arrangements occur that generate new combinations of the DNA sequences that form them.

In conclusion, the use of cloned DNA probes of varying sequence composition has shown unequivocally that each independently selected MTX-resistant cell contains an amplified unit with a unique structure. It is a necessary consequence of this finding that, at the time of formation of the unit, pieces of DNA from different parts of the genome must be joined together to form its novel structure. An understanding of where the pieces come from, how they are formed and how they are joined together in MTX-resistant cells may lead to a better understanding of the molecular rearrangements that occur "spontaneously" in many tumour cells.

Acknowledgements

We thank Ed Southern and Chris Tyler-Smith for discussions and all the staff of the MRC Mammalian Genome Unit for help during this investigation. One of us (R.C.) was supported by a Long-Term EMBO Fellowship.

*Present address: Istituto di Genetica, Università di Bari, Via Amendola 185/A, 70126 Bari, Italy.

REFERENCES

1. Alt, F.W., Kellems, R.E., Bertino, J.R. and Schimke, R.T. (1978) J. Biol. Chem. 253, 1357-1370.
2. Nunberg, J.H., Kaufman, R.J. Schimke, R.T., Urlaub, G. and Chasin, L.A. (1978) Proc. Nat. Acad. Sci. U.S.A. 75, 5553-5556.

3. Dolnick, B., Berenson, R., Bertino, J.R., Kaufman, R.J., Nunberg, J.H. and Schimke, R.T. (1979) J. Cell Biol. 83, 394-402.
4. Kaufman, R.J., Brown, P.C. and Schimke, R.T. (1979) Proc. Nat. Acad. Sci. U.S.A. 76, 5669-5673.
5. Kellems, R.E., Morhenn, V.B., Pftendt, E.A., Alt, F.W. and Schimke, R.T. (1979) J. Biol. Chem. 254, 309-318.
6. Melera, P.W., Lewis, J.A., Biedler, J.L. and Hession, C. (1980) J. Biol. Chem. 255, 7024-7028.
7. Tyler-Smith, C. and Alderson, T. (1981) J. Mol. Biol. 153, 203-218.
8. Biedler, J.L. and Spengler, B.A. (1976) Science 191, 185-187.
9. Tyler-Smith, C. and Bostock, C.J. (1981) J. Mol. Biol. 153, 237-256.
10. Schilling, J., Beverley, S., Simonsen, C., Crouse, G., Setzer, D., Feagin, J., McGrogan, M., Kohlmiller, N. and Schimke, R.T. (1982) In: Gene Amplification, Schimke, R.T. Ed. pp. 149-153, Cold Spring Harbor Lab.
11. Bostock, C.J. and Tyler-Smith, C. (1981) J. Mol. Biol. 153, 219-236.
12. Heintz, N.H. and Hamlin, J.L. (1982) Proc. Nat. Acad. Sci. U.S.A. 79, 4083-4087.
13. Bostock, C.J. and Clark, E.M. (1980) Cell 19, 709-715.
14. Bostock, C.J. and Clark, E.M., Harding, N.G.L., Mounts, P.M., Tyler-Smith, C., van Heyningen, V. and Walker, P.M.B. (1979) Chromosoma (Berl.) 74, 153-177.
15. Courtenay, V.D. and Robbins, A.B. (1972) J. Natl. Cancer Inst. 49,45-53.
16. Karn, J., Brenner, S., Barnett, L. and Cesareni, G. (1980) Proc. Nat. Acad. Sci. U.S.A. 77, 5172-5176.
17. Scalenghe, F., Turco, E.,Edstrom, J.E., Pirotta, V. and Melli, M. (1981) Chromosoma (Berl.) 82, 205-216.
18. Morrison, D.A. (1979) In: Methods in Enzymology, Wu. R., Ed., Vol. 68, pp. 326-331, Academic Press, N.Y.
19. Sillar, R. and Young, B.D. (1981) J. Histochem. Cytochem. 29, 74-78.
20. Young, B.D., Ferguson-Smith, M.A., Sillar, R. and Boyd, E. (1981) Proc. Nat. Acad. Sci. U.S.A. 78, 7727-7731.
21. Ish-Horowicz, D. and Burke, J.F. (1981) Nucl. Acids Res. 9, 2989-2998.
22. Maniatis, T., Jeffrey, A. and Kleid, D.G. (1975) Proc. Nat. Acad. Sci. U.S.A. 72, 1184-1188.
23. Southern, E.M. (1975) J. Mol. Biol. 98, 503-517.
24. Benton, W.D. and Davis, R.W. (1977) Science 196, 180-182.
25. Brison, A., Ardeshir, F. and Stark, G.R. (1982) Mol. Cell. Biol. 2, 498-507.
26. Levan, A., Levan, G. and Mitelman, F. (1977) Hereditas 86, 15-30.
27. George, D.L. and Powers, V.E. (1981) Cell 24, 117-123.
28. George, D.L. and Powers, V.E. (1982) Proc. Nat. Acad. Sci. U.S.A. 79, 1597-1601.
29. Hubbell, H.R. (1982) In: Gene Amplification. Schimke, R.T., Ed., pp. 193-198, Cold Spring Harbor Lab.