

DIHYDROFOLATE REDUCTASE GENE
AMPLIFICATION IN HUMAN CELL LINES
VA₂-B AND HELA BU25

Thesis by
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Dedicated
to
Human Biology:
Many Roads Lead to Rome

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ABSTRACT

Mammalian gene amplification has been studied using as a model system the amplification of the endogenous Dihydrofolate Reductase (DHFR) gene in the human cell lines VA₂-B and Hela BU25. Cell lines were stepwise-selected to high DHFR gene copy number using methotrexate, a DHFR enzyme inhibitor. Multiple cell lines and their derivatives were analyzed throughout the amplification process by karyotype, DHFR gene copy number, DHFR protein level, cell growth rate and, in some instances, by pulsed-field gel electrophoresis (PFGE).

Chromosomal fragmentation and rearrangements were observed in the initial stages of amplification in all cell lines examined. In subsequent stages, the formation of double minute chromosomes (DM) and Homogeneously and Abnormally Staining Regions (HSR/ASR) were observed in all cell lines except one. The order of appearance of DMs and HSRs varied among cell lines with HSRs becoming predominant at later stages under stable selection. In contrast to findings reported in other systems, DMs and HSRs were found to coexist within the same cell for extended periods of time in some cell lines.

A novel class of mammalian extrachromosomal, submicroscopic DNA elements was discovered in some cell lines through the use of PFGE. The amplified DHFR genes in one cell line, Hela 10B3, were found to reside solely in this class of DNA element ("amplisome"). Amplisomes may represent the initial, or an obligatory, molecular intermediate in the mammalian gene amplification process, at least in some cases.

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INTRODUCTION

DNA sequence amplification is a phenomenon occurring during the course of normal development (rDNA in *Xenopus* oocytes, chorion protein genes in *Drosophila melanogaster* (1,2)), as a rare de novo event in humans (110), in somatic mammalian cells cultured under conditions of selective pressure for the expression of particular genes (3) and in mammalian tumors, both inducibly and de novo (4,5). Amplification is associated with characteristic changes of the genome, including expanded chromosomal regions containing the amplified sequences known as "Homogeneously" or "Abnormally" Staining Regions (HSR/ASR) (3,6,7), with extrachromosomally located sequences known as minute or double-minute chromosomes (DM) and small chromatin bodies (SCB) (8,9) and, more recently, with submicroscopic, extrachromosomal elements or "amplisomes" (Chapter Seven,76,97).

Sequence-specific DNA amplification in cultured mammalian cells has been observed for a number of genes under selective conditions requiring increased gene expression, including dihydrofolate reductase (DHFR) (10-15), CAD gene (16), HGPRT gene (17), hydroxymethyl glutaryl COA reductase (18), adenosine deaminase (19), ornithine decarboxylase (20), metallothionein-I,II (21,22), epidermal growth factor receptor (23) and P-glycoprotein (24-27). Genes amplified in primary tumors include acetylcholinesterase and butyrylcholinesterase (109), various proto-oncogenes, such as c-myc, including choline deficient diet-induced c-myc gene amplification in rat hepatocarcinoma (28,111), n-myc (28-30,104), ki-ras (31,105) and erbB-2(neu) (32), as well as the target genes of chemotherapeutic agents such as the genes for dihydrofolate reductase (33-35) and P-glycoprotein (human MDR1): the latter is a protein

associated with multidrug resistance to anthracyclines, vinca alkaloids, epipodophyllotoxin and actinomycin D (36).

In mammalian tissue culture, stepwise selection can increase the copy number of the gene selected for several thousand fold (19). Amplified sequences reside variably in HSRs, DMs or amplisomes.

HSRs (and ASRs) are expanded chromosomal regions with aberrant trypsin-Giemsa staining properties, that can be located at or near the normal site of the amplified gene (30,37,38) or on apparently unrelated chromosomes (7,14,22). HSRs/ASRs may be small or may comprise the majority of the length of a chromosome (3,6,7). The units of amplification (amplicon) of the selected gene are generally much larger than the gene itself, with amplicons ranging in size from 200 kb to over 3,000 kb (39,40). Genes adjacent to the selected gene can be included in the amplicon and may also be expressed at high level (26,41). Recent work indicates that amplicons may be arranged in a tandem fashion or head-to-head (39), that at least a portion of them may be associated with large, inverted duplications in some systems (39,42,102,103), and that the process may involve recombination "hotspots" enriched in alu-like repeats and palindromic sequences (92,93).

Due to their association with centromeric sequences, HSRs are mitotically stable (7), although some evidence indicates that they may undergo continuous internal rearrangement (43). There is evidence to suggest that HSRs can translocate from their chromosome of origin to other chromosomes (6).

DMs are small (5,000 kb – 20,000 kb), acentromeric, self-replicating, extrachromosomal elements. DMs have been shown to replicate once during S phase in a semiconservative manner and to segregate near-randomly at mitosis. Due to the lack of centromeric sequences, amplified genes on DMs are

mitotically unstable (6,45-50). DMs characteristically vary in number between cells of a population and may also vary in size both between cells and within a single cell. DMs generally appear as paired, spherical chromatin structures in metaphase spreads, although they may be observed singly. They have also been observed to associate preferentially with the ends of chromosomes or in large accumulations within a metaphase spread (47,48).

Before this report, HSRs and DMs have been described as mutually exclusive phenomena with a cell exhibiting one or the other but not both, with rare exceptions (77,96,98). A species-specific preference has been observed, with DMs predominating in murine cell lines and with amplification occurring exclusively via HSRs in hamster (3). The significance and mechanistic implications of this species-specific bias is not clear.

Amplisomes are recently described acentric, self-replicating, extrachromosomal elements ranging in size from 200 Kb - 750 Kb (Chapter Seven, 76,97,98). Amplisome-mediated gene amplification has been demonstrated for c-myc in two human tumor cell lines (116), for endogenous DHFR and MDR1 genes in drug-selected human cell lines (Chapter Seven, 97,107) and for transfected CAD genes in a Chinese hamster ovary cell line (76). Evidence suggests that the latter CAD amplisomes may possibly evolve into DMs, and even HSRs, over time (106). Amplisomes, unlike DMs and HSRs, have not yet been demonstrated in tumors, *in vivo*.

The generative mechanism of DNA sequence amplification in mammalian systems is not known. Suggested models include chromosome fragmentation (6), unequal sister chromatid exchange (51,52), onionskin reinitiation overreplication (53,54), yeast 2-micron element-like overreplication (extrachromosomal double rolling circle replication) (55) and intrachromosomal double rolling circle

replication (93). Evidence from rDNA sequence amplification in *Drosophila* supports unequal sister chromatid exchange in this system (56), while rDNA amplification in *Xenopus* oocytes appears to occur via rolling-circle replication of extrachromosomal, circular DNA sequences (57). Further, chorion protein gene amplification in *Drosophila* has been demonstrated to result from onionskin overreplication, with multiple reinitiation at replication origins apparently controlled by a specific DNA control element (58,59).

Evidence in mammalian systems is not so clear. The large size of the amplified units has hindered attempts to reach definitive molecular conclusions. Studies on amplified transfected genes have suggested crossing-over and/or burst-replication of core sequences (60), however, the extension of these results to the large amplicons resulting from native sequence amplification has not been shown. Unequal sister chromatid exchange events sufficient to generate HSRs would have to occur many times over successive cell generations and would result in a reciprocal gain-loss of sequences between sister chromatids. This would be evidenced by an expanding HSR region on one homologue and a reduction or loss of the corresponding region on the other, a result which has not been reported. Similarly, no definitive evidence exists to support onionskin overreplication in mammalian systems and reports on the ability of specific DNA sequences to undergo multiple rounds of replication within a single cell cycle are conflicting (61,62). Recently, the formation of large, inverted duplications have been implicated in association with gene amplification in at least some systems (42,102,103) and a chromosomal excision event of *n-myc* (supportive of models invoking recombination across a replication loop) has been reported in a human neuroblastoma cell line with amplified *n-myc* sequences (108).

Pretreatment of cultured cells with hydroxyurea (63), the tumor promoter TPA (64), UV irradiation (65) and other agents (66) have been shown to increase the frequency with which cells become resistant upon selection. The significance of these results to the amplification processes in tumors is uncertain. In general, tumors and tumor cell lines have demonstrated drug-selected gene amplification events at frequencies about $10^3 - 10^4$ that of primary diploid cells, indicating an inherent difference in the genetic stability of tumors and tumor cell lines versus normal cells (114,115). The extension of these data to normal, in vivo stem cells (the postulated precursors of tumors) remains to be demonstrated. Similarly, the in vivo significance of the loss of amplified c-myc genes in the human HL-60 cell line (112) and the loss of transfected oncogenes in mouse NIH 3T3 cells (113) that is induced by poly (ADP-ribose) polymerase inhibitors, in vitro, has yet to be demonstrated.

Lastly, the relationship, if any, of the small, polydisperse, circular DNA (SPC DNA) found ubiquitously in tissues (94,95) and tissue culture systems in many species (67), and of its generative mechanism, to the processes of sequence-specific amplification in mammalian cells is unknown.

This report describes studies carried out on cultured human cells that have become resistant to the anticancer agent, methotrexate, via dihydrofolate reductase (DHFR) gene amplification. Methotrexate, a folate analog, is transported into the cell by a specific transport system (68) and binds irreversibly to DHFR, resulting in inhibition of purines, thymidilic acid and glycine synthesis. Methotrexate-induced DHFR gene amplification has been reported in murine (5,6), hamster and human cell lines (5,6,13) and in human tumors (33-35). The present report addresses the questions of the chromosomal location of the normal human DHFR gene and its related pseudogenes and of the karyotypic

abnormalities associated with its amplification in derivatives of the human cell lines Hela S3 and VA₂-B. Further, a novel approach has been employed to isolate in pure form and partially characterize a fraction of the amplified DHFR genes that exist extrachromosomally as submicroscopic elements in several of the cell lines studied.

It is hoped that this report will lead to a greater understanding of the phenomenon of sequence-specific mammalian gene amplification, in general, and to possible insights into the development and clinical treatment of human cancers resistant to chemotherapeutic agents.

CHAPTER ONE

**Human Dihydrofolate Reductase Gene is Located in Chromosome 5
and is Unlinked to the Related Pseudogenes**

Human dihydrofolate reductase gene is located in chromosome 5 and is unlinked to the related pseudogenes

(human-mouse cell hybrids/DNA transfer hybridization/cDNA/diphtheria toxin sensitivity)

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ABSTRACT The chromosomal location of the human dihydrofolate reductase (DHFR; EC 1.5.1.3) gene that is amplified in a methotrexate-resistant human cell line has been investigated by screening a large number of human-mouse cell hybrids containing overlapping subsets of human chromosomes. A correlation of genomic blotting data with the chromosome constitution of the individual cell hybrids has allowed the assignment of the human *DHFR* gene to chromosome 5. This chromosome assignment has been confirmed by the observation of a concomitant loss of the human *DHFR* gene and of sensitivity to diphtheria toxin, a marker associated with chromosome 5, in two human-mouse cell hybrids selected for resistance to the toxin. Six *EcoRI* fragments of human DNA containing *DHFR* pseudogenes or other *DHFR*-related sequences have been assigned to chromosomes other than chromosome 5.

Recently, several cell variants resistant to high concentrations of the folate antagonist methotrexate (MTX) have been isolated from two different human cell lines, HeLa BU25 and VA₂-B (1). As described for other mammalian culture systems (2), these cell lines overproduce dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate:NADP⁺ oxidoreductase, EC 1.5.1.3), the target enzyme of MTX (1), as a consequence of selective amplification of the structural gene for DHFR (3). A striking feature of these MTX-resistant human cell variants is the pleomorphism of chromosomal alterations they exhibit. These include a highly variable number of double-minute chromosomes (DMs) (1), a homogeneously staining region (HSR) in one or more marker chromosomes, and a duplicated set of chromosomes in most cells of some variants (1). To understand the nature and origin of these chromosomal anomalies, the normal chromosomal location of the *DHFR* structural gene must be defined, since, in rodent cells (2) and human cells (unpublished data), both DMs and HSRs have been shown to be the sites of amplified *DHFR* genes. The recent cloning and characterization of human *DHFR* cDNAs (4) and the identification of the amplified *DHFR* structural gene fragments in *EcoRI* digests of genomic DNA (3) make it possible to study the segregation of the human *DHFR* gene in human-mouse hybrid cells containing overlapping subsets of human chromosomes and, by this approach, to identify the chromosome in normal human cells that is the site of the *DHFR* gene. In this study, the human *DHFR* gene is assigned to chromosome 5, and six *EcoRI* fragments containing *DHFR* pseudogenes or other *DHFR*-related sequences are shown to be located in human chromosomes other than 5.

MATERIALS AND METHODS

Materials. The restriction enzyme *EcoRI* was obtained from New England BioLabs or Bethesda Research Laboratories, *Escherichia coli* DNA polymerase I from New England Nuclear, agarose from Sigma, dextran sulfate from Pharmacia, nitrocellulose paper from Schleicher & Schuell, and [α -³²P]dCTP from Amersham.

Cell Lines and Conditions of Growth. The human cell lines VA₂-B and HeLa S3 were grown as described (1). The mouse cell line A9 was grown in Eagle's phosphate medium supplemented with 5% calf serum and azaguanine at 3 μ g/ml. The parents of the hybrid cell lines of the AHA series were mouse A9 and human GM144; those of the 41pT series and of the FRY series, mouse A9 and human GM126; and those of the BDA series, mouse A9 and human GM589. The human cell lines GM144 and GM126 are two fibroblast lines with balanced chromosome translocations not involving chromosome 5, and the cell line GM589 is a fibroblast line with a balanced translocation of almost the entire chromosome 5 onto the long arm of chromosome 14 (Human Genetic Mutant Cell Repository, Camden, NJ, 1982). Nine independent hybrid clones, five subclones isolated from them by dilution plating, and two diphtheria toxin-resistant sublines derived from two independent clones were analyzed. The hybrids were grown as described (5).

DNA Extraction. High molecular weight DNA from VA₂-B, HeLa S3, and A9 cells was prepared by the method of Gross-Bellard *et al.* (6). Cell hybrid DNAs were prepared as reported (5), additionally digested with RNase and Pronase, extracted with phenol, precipitated with ethanol, and suspended in 10 mM Tris-HCl/1 mM EDTA, pH 8.0.

Preparation of Radioactive Probes. Nick-translation of the insert fragment of pHD84 (4), of the "probe 1.7," a derivative of pAT153 containing a 1.14-kilobase (kb) chromosome 5-specific single-copy DNA fragment (provided by P. Pearson), was carried out as described (4), using DNase I at 25 pg/ml, 4 units of *E. coli* DNA polymerase I, and 75–125 μ Ci (1 Ci = 37 GBq) of [α -³²P]dCTP (2000–3000 Ci/mmol) per 100 ng of DNA, with the other unlabeled dNTPs at 20 μ M, to give a specific activity of 4–8 $\times 10^8$ cpm/ μ g. The *Bam*HI fragments *e* and *f* of the *c-fms* human oncogene (7), which is located on human chromosome 5 (7, 8), were nick-translated by a similar procedure.

DNA Transfer and Hybridization Techniques. DNA samples (amounts specified in figure legends) were digested to completion with an excess of *EcoRI* restriction enzyme, electrophoresed through a 0.7% agarose slab gel, and transferred to nitrocellulose filters by the method of Southern (9).

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Abbreviations: MTX, methotrexate; DHFR, dihydrofolate reductase; DM, double-minute chromosome; HSR, homogeneously staining region; kb, kilobase(s).

Hybridization with the ^{32}P -labeled probe was carried out as described (3), except for the absence of poly(cytidylic acid), using $1.5\text{--}2.0 \times 10^7$ cpm of probe per filter in 5 ml of hybridization buffer. The filters were washed at 68°C once with $6\times \text{NaCl/Cit}$ ($1\times \text{NaCl/Cit} = 0.15 \text{ M NaCl}/0.015 \text{ M sodium citrate}$)/ $10\times$ Denhardt's solution (10)/0.1% sodium pyrophosphate/0.1% NaDodSO₄ for 30 min, three times with $2\times \text{NaCl/Cit}/0.1\%$ NaDodSO₄, and three times with $0.5\times \text{NaCl/Cit}/0.1\%$ NaDodSO₄, 20 min each. The filters were then dried and exposed for autoradiography (4).

Isozyme and Karyotype Analysis. All clones and subclones were subjected to isozyme analysis and most were karyotyped; furthermore, the DNA from the majority of the hybrids was also tested with chromosome 5-specific probes (*Bam*HI fragments *e* and *f* of the *c-fms* human oncogene and "probe 1.7") and with a chromosome 12-specific probe (5). The isozyme tests were previously described (5). The chromosome constitution of the hybrids was determined on 25–50 Giemsa-banded metaphases per hybrid. The following criteria were used for characterization of the cell hybrid lines: A cell line was scored positive (+) for a human chromosome when its presence was revealed by appropriate isozyme expression and by karyotypic identification in $>20\%$ of metaphases; in some cases, a clear karyotypic identification alone or isozyme expression in the absence of karyotype data was used for positive chromosome scoring. When no isozyme markers were expressed and when, in the karyotyped hybrids, $<5\%$ of cells exhibited the specific human chromosome, the hybrid was scored negative (–). In the case of chromosome 5, the results of the specific probe hybridization assays were also used as criteria, and they were always found to be in agreement with the other scoring criteria, so that all scorings for chromosome 5 were based on at least two criteria. A hybrid was scored as weakly positive (+/–) when the chromosome was present in 5–20% of metaphases and the isozyme analysis gave inconsistent results in multiple assays. In cases in which the isozyme and karyotype data were insufficient to clearly characterize a hybrid, this was scored 0 and the corresponding data were not included in the mapping summary. In the hybrids of the series BDA deriving from A9 and the human fibroblast line GM589 that were positive for chromosome 5 by karyotypic analysis, both translocated and nontranslocated chromosomes 5 were observed and scored. In three of these hybrids (10a3-1, 10a3-4, and 10a3-6), the translocated chromosomes 5 were predominant (83–100%), in two other hybrids (17b17 and 10a4aFa_{9.1}), mostly nontranslocated chromosomes 5 were observed (79% and 63%, respectively).

RESULTS

Organization of *DHFR*-Specific Sequences in Genomic DNAs from Human and Mouse Cells. The plasmid pH84 is a human *DHFR* cDNA clone derived from the MTX-resistant VA₂-B derivative 6A3 and contains the complete human *DHFR*-encoding sequence (3, 4). The coding sequence of human *DHFR* shows an 89% nucleotide sequence homology to that of the mouse (11). The plasmid pH84 was used to screen human–mouse somatic cell hybrid DNAs for human *DHFR*-specific sequences. Southern blots of *Eco*RI-digested human genomic DNA probed with pH84 show a complex pattern consisting of fragments of the human *DHFR* gene that is amplified in VA₂-B 6A3 cells and of fragments containing other *DHFR*-specific sequences (3). Two of the latter fragments have been shown to contain portions of an intronless pseudogene (3), while the nature of the other *DHFR*-related sequences is uncertain. In this paper, fragments that hybridize to the *DHFR* pH84 probe, but that do not belong to the structural gene amplified in 6A3 cells, will be referred to as *DHFR*-related sequences.

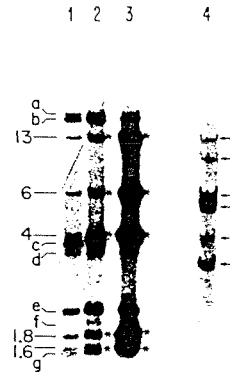


FIG. 1. Patterns of hybridization with a human *DHFR* probe of *Eco*RI-digested DNA from human and mouse cell lines. DNA samples (10 μg) were digested to completion with *Eco*RI, electrophoresed through agarose slab gels, transferred to nitrocellulose paper, and hybridized with the ^{32}P -labeled insert of pH84. The numbers on the left indicate the normal human *DHFR* gene fragments (also marked by asterisks) and represent their sizes in kb, and the letters refer to DNA fragments containing human *DHFR*-related sequences. The long arrows on the right of lane 4 indicate the mouse *DHFR* gene fragments; the short arrows refer to DNA fragments containing mouse *DHFR*-related sequences. The band corresponding to the upper fragment containing mouse *DHFR*-related sequences appears with variable intensity in different blots and may represent a fragment particularly resistant to *Eco*RI. Lane 1, GM589; lane 2, HeLa S3; lane 3, VA₂-B; lane 4, A9.

Fig. 1 illustrates the distribution of *DHFR*-specific sequences that hybridize to the pH84 probe in *Eco*RI digests of genomic DNA from the normal human fibroblast line GM589 (lane 1), HeLa S3 (lane 2), and VA₂-B, a human cell line containing a moderate amplification of the human *DHFR* gene (3) (lane 3). The fragments of the structural gene amplified in VA₂-B DNA, which correspond to those amplified in the DNA from the MTX-resistant VA₂-B 6A3 cell line (3), are 13, 6, 4, 1.8, and 1.6 kb in size. These fragments, which span the entire reading frame for human *DHFR*, have recently been shown to be a part of the human structural gene for *DHFR* by molecular cloning and DNA sequence analysis (12). The 1.6-, 4-, and 13-kb *Eco*RI fragments have been cloned, and the organization of this portion of the gene has been determined (13). Fragments not associated with the structural gene are designated in Fig. 1 by letters *a*–*g* and have molecular sizes of 18, 17, 3.8, 3.5, 2.3, 2, and ≈ 1.6 kb, respectively (ref. 4 and present work). The two fragments *d* and *e* contain the recently isolated intronless pseudogene (3) mentioned above.

The hybridization pattern of an *Eco*RI digest of mouse A9 cell DNA probed with the human *DHFR* probe pH84 is shown in lane 4 of Fig. 1. The four expected *Eco*RI fragments of the mouse *DHFR* gene can be seen (14), as indicated by long arrows. In addition, two previously unreported mouse DNA fragments containing *DHFR*-related sequences are detected by the human *DHFR* probe, as shown by short arrows. The fragments of the human *DHFR* gene with sizes of 13, 6, and 4 kb migrate to positions in the gel that correspond closely to those of mouse DNA fragments hybridizing with the *DHFR* probe. In contrast, the 1.8- and 1.6-kb gene fragments are found in regions of the blot free of mouse-specific bands. Thus, it has been possible to score the human–mouse hybrid DNAs for the human *DHFR* gene by using the 1.8- and 1.6-kb bands as diagnostic of the entire gene. How-

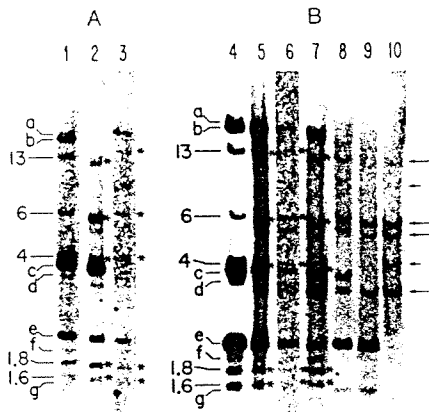


FIG. 2. Patterns of hybridization with a human *DHFR* probe of *EcoRI* digested DNA from human-mouse cell hybrid clones. DNA samples [10 μ g, except BDA 10a3-4 and BDA 10a3-6 (\approx 2 μ g)] digested with *EcoRI* were treated as described for Fig. 1. (A) Lane 1, GM589, human parental line; lane 2, BDA 17b17; lane 3, BDA 10a3-6. (B) Lane 4, HeLa S3; lane 5, BDA 10a3-1; lane 6, BDA 10a3-4; lane 7, BDA 10a4aFa₉₋₁; lane 8, BDA 14b25; lane 9, BDA 14b25-2; lane 10, A9, mouse parental line. For an explanation of numbers, letters, and arrows, see legend to Fig. 1. The asterisks indicate the human *DHFR* gene fragments in the cell hybrid DNAs.

ever, bands corresponding to the other expected human *DHFR* gene fragments were always observed whenever the 1.8- and 1.6-kb bands were present. Furthermore, in several cases, from the intensity of the signal given by these other bands (in particular the 13- and 6-kb bands) and/or the absence of expected mouse gene fragments, the association of these bands with human gene fragments could be inferred. One also sees in Fig. 1 that the human DNA fragments containing *DHFR*-related sequences *a*, *b*, *c*, *e*, *f*, and *g* are not obscured by cross-hybridizing mouse DNA fragments.

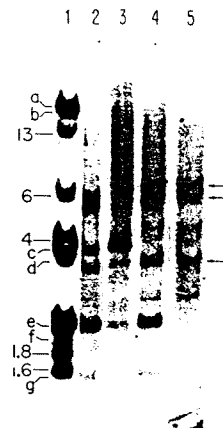


FIG. 3. Patterns of hybridization with a human *DHFR* probe of *EcoRI*-digested DNA from human-mouse hybrid cell DNAs. DNA samples (10 μ g) digested with *EcoRI* were treated as described for Fig. 1. Lane 1, HeLa S3; lane 2, AHA 16e; lane 3, AHA 3d-2; lane 4, FRY-1; lane 5, A9, mouse parental line. For an explanation of numbers, letters, and arrows, see legend to Fig. 1.

Analysis of the Human *DHFR* Gene and Related Sequences in Human-Mouse Cell Hybrid DNAs. Southern blots of *EcoRI*-digested DNA from several human-mouse cell hybrids probed with pHD84 are shown in Figs. 2 and 3. In almost all cell hybrid DNAs shown, the characteristic bands of the mouse *DHFR* gene can be seen. By contrast, the fragments of the human structural gene for *DHFR* (asterisks) are present in only 5 of the 10 human-mouse DNA samples shown. Two hybrids, BDA10a3-4 and BDA10a3-6, gave weak hybridization signals, due to the lower amounts of DNA available. However, the 1.8- and 1.6-kb bands were clearly discernible (especially on the original autoradiogram); furthermore, the 13- and 6-kb bands, because of their intensity, could be assigned with reasonable confidence to human *DHFR* gene fragments. In all blots, one or more fragments with human *DHFR*-related sequences are also observed. The results of genomic blotting and chromosome

Table 1. Human chromosome distribution in human-mouse cell hybrids and summary of genomic blotting results

Hybrid cell line	Reaction of genomic <i>EcoRI</i> fragments with pHD84*							Presence of human chromosome. as determined by human isozyme expression and by cytogenetic analysis†																								
	<i>DHFR</i>	<i>a</i>	<i>b</i>	<i>c</i>	<i>d/e</i>	<i>f</i>	<i>g</i>	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X		
BDA14b25	-	0	-	+	0/+	-	+	+	-	+	+	-	-	+	0	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	
BDA14b25-2	-	0	-	-	0/+	-	+	-	-	+	+	-	-	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+	
BDA10a3-1	+	+	+	+	+/+	+	0	-	+	+	-	+	+	+	+	+	+	+	+	-	-	-	+	+	-	+	-	+	-	-	+	
BDA17b17	+	+	+	+	0/+	0	0	+	-	+	+	+	+	-	-	-	+	-	+	-	+	+	-	-	+	+	+	+	+	+	+	
FRY-1	-	-	-	-	0/+	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	
41pT2a‡	-	+	+	+	+/+	0	+	-	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	
BDA10a3-4	+	0	+	+	+/+	0	0	-	+	+	0	+	+	+	0	-	+	-	+	0	-	-	0	0	-	-	+	-	0	+	+	
BDA10a3-6	+	0	0	0	0/+	0	0	-	+	+	0	+	+	+	0	-	+	-	+	0	-	-	0	0	-	-	+	-	0	+	+	
AHA3d2	-	-	-	-	0/+	0	+	-	-	+	+	-	0	0	-	+	+	+	+	-	-	-	-	-	+	+	-	+	-	0	+	
AHA16e	-	-	-	+	0/+	-	+	-	-	0	-	+/+	-	-	0	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	
AHA11a‡	-	-	-	-	0/-	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	
BDA10a4aFa ₉₋₁	+	0	+	+	+/+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	-	-	-	-	-	-	-	+	
BDA10a3	+	+	+	+	0/+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	-	-	-	-	+	
BDA10a3DT	-	+	+	+	0/+	+	+	-	+	0	-	+	+	+	+	0	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
BDA17b17-1	+	0	0	+	0/+	-	+	+/+	+/+	0	+	+	+	+	0	-	-	+	+/+	+	+	-	+	-	-	+	+	+	+	+	+	
BDA17b17-1DT	-	0	0	+	0/+	-	+	+/+	-	0	+	+	-	-	-	0	-	-	-	+	+	-	0	-	-	-	-	-	+	+	+	

*+, Fragment(s) present; -, fragment(s) absent; 0, undetermined.

†See *Materials and Methods* and ref. 5 for the techniques of isozyme and karyotype analysis.

‡Genome blots not shown.



FIG. 4. Correlation of resistance to diphtheria toxin with the loss of the human *DHFR* gene (A) and the loss of DNA sequences complementary to a human chromosome 5-specific probe (B) in two human-mouse cell hybrids. Two human-mouse cell hybrid clones were grown in the presence of diphtheria toxin and a toxin-resistant subline was derived from each (see text). (A) DNA samples (10 µg) digested with *EcoRI* were treated as described for Fig. 1. Lane 1, HeLa S3; lane 2, BDA 10a3; lane 3, BDA 10a3DT, toxin-resistant; lane 4, BDA 17b17-1; lane 5, BDA 17b17-1DT, toxin-resistant; lane 6, A9. Normal *DHFR* gene fragments in lanes 2 and 4 are indicated by asterisks. (B) DNA samples (10 µg) digested with *EcoRI* and transferred to nitrocellulose paper were hybridized with ³²P-labeled probe 1.7 specific for human chromosome 5 (see text). Lane 7, HeLa S3; lane 8, BDA 10a3; lane 9, BDA 10a3DT, toxin-resistant; lane 10, BDA 17b17-1; lane 11, BDA 17b17-1DT, toxin-resistant; lane 12, A9.

analysis for these and other cell hybrids described below are detailed in Table 1. The data in Table 1 strongly suggest that chromosome 5 contains the *DHFR* gene (see Discussion). The results of Table 1 also indicate that none of the DNA fragments containing human *DHFR*-related sequences segregate with chromosome 5.

Correlation of Human *DHFR* Structural Gene, Chromosome 5 Markers, and Diphtheria Toxin Sensitivity in Human-Mouse Cell Hybrids. The assignment of the human *DHFR* gene to chromosome 5 indicated by the mapping experiments discussed above suggested a confirmatory experiment based upon the selectable loss of a marker previously

mapped in human chromosome 5. It has been shown that sensitivity to diphtheria toxin in human-mouse cell hybrids is associated with a gene or genes located in human chromosome 5 (15). Therefore, human-mouse cell hybrids can be selected for the loss of human chromosome 5 by growing them in the presence of diphtheria toxin. Two human-mouse cell hybrids containing the human *DHFR* gene were exposed to Connaught diphtheria toxin at 2×10^{-5} L_t units/ml (15), and a toxin-resistant subline was isolated from each. The four lines were subjected to isozyme and karyotype analysis (Table 1); in particular, they were screened for expression of hexosaminidase B, a human chromosome 5 marker (16), and further tested for the presence of chromosome 5 by probing genomic blots with a human chromosome 5 unique sequence DNA marker (probe 1.7). By both criteria, the two cell lines had retained human chromosome 5 while the two diphtheria toxin-resistant derivatives had lost this chromosome. It should be noticed that chromosome 5 was the only chromosome lost by both toxin-resistant sublines. The DNAs from the four lines were then screened for the human *DHFR* structural gene. Fig. 4 shows the results of the blotting experiment utilizing the human *DHFR* probe (Fig. 4A, lanes 2–4) or the chromosome 5-specific probe 1.7 (Fig. 4B, lanes 8–11). The absence of the 1.8- and 1.6-kb human *DHFR* bands from the DNA blots of the diphtheria toxin-resistant lines indicates that the human *DHFR* gene has been lost. Thus, the loss of chromosome 5 in the diphtheria toxin-resistant lines is accompanied by the loss of the human *DHFR* gene. This result strongly supports the assignment of the human *DHFR* gene to chromosome 5.

Polymorphism of the probe 1.7 in human populations has been reported (17). This is reflected in the appearance of additional probe 1.7-related bands in HeLa cell DNA (Fig. 4B, lane 7) and in the DNA from the cell hybrid BDA 17b17-1 (Fig. 4B, lane 10).

DISCUSSION

The distribution of the human *DHFR* gene in all the hybrid cell lines analyzed, including the diphtheria toxin-resistant derivatives, is summarized in Table 2. It is clear that chromosome 5 is the only chromosome showing a complete concordance of segregation with the *DHFR* gene. Other genomic blotting data (not shown) have revealed the absence of the *DHFR* gene in six additional human-mouse cell hybrids lacking chromosome 5. We conclude that the *DHFR* structural gene that has been amplified in the human cell lines VA₃-B and VA₂-B 6A3 resides in human chromosome 5.

The present analysis has also provided information concerning the chromosomal location of several fragments containing human *DHFR*-related sequences. Table 3 summarizes the most probable chromosome assignment of each of the *EcoRI* fragments b–g. Although the available data do not

Table 2. Assignment of the structural gene for *DHFR* to human chromosome 5

Segre- gation*	Number of hybrids showing concordant or discordant segregation of human <i>DHFR</i> gene with each chromosome																						
	1	2	3	4	5†	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
Concordant																							
+ / +	1	5	6	4	7	5	0	3	1	5	3	6	2	2	1	2	3	2	2	5	2	2	7
- / -	6	7	2	4	8	7	7	4	5	5	5	5	5	6	8	6	7	5	6	7	6	4	1
Discordant																							
+ / -	5	1	0	1	0	2	7	2	5	2	4	0	3	5	6	3	2	5	5	2	5	3	0
- / +	2	2	4	5	0	2	1	3	1	4	4	4	4	3	1	2	2	4	3	2	3	4	8
Total	7	3	4	6	0	4	8	5	6	6	8	4	7	8	7	5	4	9	8	4	8	7	8

Only the unambiguous data of Table 1 (+ or -) were used in the compilation of this table.

*In each case the first plus or minus refers to the presence or absence, respectively, of the human *DHFR* gene, as determined by genomic blotting experiments. The second plus or minus refers to the presence or absence of the chromosome numbered on the top of the table, as determined by isozyme and karyotype data.

†Complete concordance of segregation between the *DHFR* structural gene and human chromosome 5 indicates that the structural gene resides on this chromosome.

allow an unambiguous chromosome assignment for these fragments, they do exclude most chromosomes, including chromosome 5, as their sites. Two of these fragments, *d* and *e* (Fig. 1), have recently been shown to contain an intronless *DHFR* pseudogene, $\psi HD1$ (3). On the basis of the data reported here, an assignment of the pseudogene $\psi HD1$ to chromosome 3 or X has been made, with the evidence strongly favoring chromosome 3. Two other fragments, *b* and *c* (Fig. 1), because of their size and the intensity of the hybridization signal, appear to be good candidates for containing two additional intronless *DHFR* pseudogenes that have recently been isolated from human DNA libraries (13). In particular, fragment *b* has a size (≈ 17 kb) that is compatible with the >15 -kb *EcoRI* subfragment carrying the *hDHFR- $\psi 2$* pseudogene isolated from a fetal DNA library, and fragment *c* has a size (≈ 3.8 kb) very close to that of the fragment containing the *hDHFR- $\psi 1$* pseudogene isolated from an adult DNA library. The present analysis indicates that fragment *b* is located in chromosome 2 or 6, while fragment *c* is located in chromosome 3 or X.

The nature of the other fragments producing faint bands in the *EcoRI* restriction pattern of *DHFR*-specific sequences in human genomic DNA (fragments *a*, *f*, and *g*, Fig. 1) is unknown. The low intensity of the signals given by these fragments with a *DHFR* cDNA probe and the chromosome segregation pattern would exclude the possibility that they represent *DHFR* genes with different *EcoRI* restriction patterns.

The present chromosome assignment of the human *DHFR* gene is relevant to the origin of the chromosomal anomalies, DMs and HSRs, observed in several MTX-resistant cell lines (1, 18, 19). The human MTX-resistant cell line VA₂-B 6A3 exhibits an HSR containing amplified *DHFR* genes in the long arm of a chromosome clearly distinct from chromosome 5 (unpublished data). HSRs observed in other human MTX-resistant cell lines have been reported to be located in chromosomes 6, 19 (ref. 18), and 10 (ref. 19). The observation in human MTX-resistant cell lines of HSRs containing amplified *DHFR* genes in chromosomes other than chromosome 5 strongly suggests that a translocation event from chromosome 5 to other chromosomes has occurred in these cell lines. A role for DMs in the amplification and translocation of the *DHFR* gene has been suggested (2, 20), but very little evidence has been reported about their possible involvement. Recently, an apparent translocation and amplification of the *c-myc* gene from its normal position in human chromosome 8 to HSRs in the X chromosome has been described in malignant neuroendocrine cells derived from a human colon carcinoma (21). In the present case, an interesting possibility is suggested by the observation that the human genome contains, besides the *DHFR* structural gene that is amplified in 6A3 cells, up to as many as six other loci showing homology to the human *DHFR* probe. It is conceivable that translocation events involving homologous recombination between the *DHFR* structural gene in chromosome 5 and other

DHFR-related sequences located elsewhere in the genome are responsible for the formation of at least some of the HSRs observed in MTX-resistant cell lines.

Finally, the chromosomal linkage between the *DHFR* gene and the gene(s) conferring diphtheria toxin sensitivity to human cells parallels the demonstrated presence of these two loci in chromosome 2 of Chinese hamster cells (22–24). It should also be mentioned that three other genes, *leuS*, *emtB*, and *chr*, are linked to the *DHFR* gene in both human chromosome 5 and Chinese hamster chromosome 2 (25). Considering the distance between these loci, the above observations provide a striking example of conservation of gene organization among mammalian species.

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1. Masters, J., Keeley, B., Gay, H. & Attardi, G. (1982) *Mol. Cell. Biol.* 2, 498–507.
2. Schimke, R. T., ed. (1982) *Gene Amplification* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
3. Masters, J. N., Yang, J. K., Cellini, A. & Attardi, G. (1983) *J. Mol. Biol.* 167, 23–36.
4. Morandi, C., Masters, J. N., Mottes, M. & Attardi, G. (1982) *J. Mol. Biol.* 156, 583–607.
5. Ryan, J., Barker, P. E., Shimizu, K., Wigler, M. & Ruddle, F. H. (1983) *Proc. Natl. Acad. Sci. USA* 80, 4460–4463.
6. Gross-Bellard, M., Oudet, P. & Chambon, P. (1973) *Eur. J. Biochem.* 36, 32–38.
7. Roussel, M. F., Sherr, C. J., Barker, P. E. & Ruddle, F. H. (1983) *J. Virology* 48, 770–773.
8. Groffen, J., Heisterkamp, N., Spurr, N., Dana, S., Wasmuth, J. J. & Stephenson, J. R. (1983) *Nucleic Acids Res.* 11, 6331–6339.
9. Southern, E. M. (1975) *J. Mol. Biol.* 98, 503–517.
10. Denhardt, D. T. (1966) *Biochem. Biophys. Res. Commun.* 23, 641–646.
11. Masters, J. N. & Attardi, G. (1983) *Gene* 21, 59–63.
12. Yang, J. K., Masters, J. N. & Attardi, G. (1984) *J. Mol. Biol.*, in press.
13. Chen, M.-J., Shimada, T., Davis Moulton, A., Harrison, M. & Nienhuis, A. W. (1982) *Proc. Natl. Acad. Sci. USA* 79, 7435–7439.
14. Crouse, G. F., Simonsen, C. C., McEwan, R. N. & Schimke, R. T. (1982) *J. Biol. Chem.* 257, 7887–7897.
15. Creagan, R. P., Chen, S. & Ruddle, F. H. (1975) *Proc. Natl. Acad. Sci. USA* 72, 2237–2241.
16. Gilbert, F., Kucherlapati, R., Creagan, R., Murnane, M. J., Darlington, G. J. & Ruddle, F. H. (1974) *Cytogenet. Cell Genet.* 13, 93–95.
17. Skolnik, M. H., Willard, H. F. & Menlove, L. A. (1984) *Cytogenet. Cell Genet.* 37, 210–273.
18. Bertino, J. R., Srimatkandada, S., Engel, D., Medina, W. A. & Scheer, D. I. (1982) in *Gene Amplification*, ed. Schimke, R. T. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 23–27.
19. Wolman, S. R., Craven, M. L., Grill, S. P., Domin, B. A. & Cheng, Y.-C. (1983) *Proc. Natl. Acad. Sci. USA* 80, 807–809.
20. Bostock, C. J. & Tyler-Smith, C. (1982) in *Gene Amplification*, ed. Schimke, R. T. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 15–21.
21. Alitalo, K., Schwab, M., Lin, C. C., Varmus, H. E. & Bishop, J. M. (1983) *Proc. Natl. Acad. Sci. USA* 80, 1707–1711.
22. Roberts, M., Melera, P. W., Davide, J. P., Hart, J. T. & Ruddle, F. H. (1983) *Cytogenet. Cell Genet.*, in press.
23. Roberts, M. & Ruddle, F. H. (1980) *Exp. Cell Res.* 127, 47–54.
24. Worton, R., Duff, C. & Flintoff, W. (1981) *Mol. Cell. Biol.* 1, 330–335.
25. Dana, S. & Wasmuth, J. J. (1982) *Somatic Cell Genet.* 8, 245–269.

Table 3. Mapping of *DHFR* structural gene, *DHFR* pseudogenes, and other *DHFR*-related sequences in the human genome

<i>EcoRI</i> band* (ref.)	Molecular size, kb	Chromosome
<i>DHFR</i> fragment	13, 6, 4, 1.8, 1.6	5
<i>b</i> (<i>hDHFR-$\psi 2$</i>) (13)	17	2 or 6
<i>c</i> (<i>hDHFR-$\psi 1$</i>) (13)	3.8	3 or X
<i>d/e</i> ($\psi HD1$) (4)	3.5/2.3	3 or X†
<i>f</i>	2	2 or 6
<i>g</i>	≈ 1.6	3 or X

*See Fig. 1.

†Since band *e* is present in equal quantities in human male and female placental DNAs (data not shown), the most probable chromosome is no. 3.

CHAPTER TWO

Assignment of Human Dihydrofolate Reductase Gene to Band q23 of Chromosome 5 and of Related Pseudogene Psi-HD1 to Chromosome 3

Assignment of Human Dihydrofolate Reductase Gene to Band q23 of Chromosome 5 and of Related Pseudogene Ψ HD1 to Chromosome 3

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Abstract—The chromosomal location of the human dihydrofolate reductase (*DHFR*; EC 1.5.1.3) gene that is amplified in a methotrexate-resistant human cell line has been investigated by screening a number of human–Chinese hamster ovary cell hybrids containing terminal and interstitial deletions in human chromosome 5. A correlation of genomic blotting data with the chromosome 5 constitution of the individual hybrids has allowed the assignment of the human *DHFR* gene to 5q23. The present work also establishes the location of the related intronless pseudogene Ψ HD1 in chromosome 3.

INTRODUCTION

Dihydrofolate reductase (*DHFR*; 5,6,7,8-tetrahydrofolate: NADP oxidoreductase, EC 1.5.1.3) is the target enzyme of the folate antagonist methotrexate (MTX). Stepwise selection in the presence of MTX has resulted in the amplification of the *DHFR* gene, with the formation of double-minute (DM) chromosomes and/or of a homogeneously staining region (HSR) in one or more chromosomes, in both rodent and human cell cultures (1–3). The recent cloning and characterization of human *DHFR* cDNAs (4) and the identification of the *DHFR* structural gene fragments in *EcoRI* digests of genomic DNA (3) has made it possible to study the segregation of the human *DHFR* gene in human–mouse hybrid cells containing overlapping subsets of human chromosomes and has allowed its assignment to chromosome 5 (5). In the present study, a series of human–

Chinese hamster ovary (CHO) cell hybrids containing terminal and interstitial deletions in human chromosome 5 have been examined in order to determine the location of the *DHFR* gene within this chromosome. This analysis has allowed the assignment of the human *DHFR* gene to 5q23 and has confirmed the previously tentative assignment of the *DHFR* pseudogene Ψ HD1 (3) to chromosome 3 (5).

MATERIALS AND METHODS

Hybrid Cell Lines and Segregants. The isolation and characterization of interspecific cell hybrids between normal human leukocytes and the Chinese hamster ovary (CHO) cell line UCW 56, which retain human chromosome 5 under selective pressure, was described previously (6). More recently, the isolation of a series of segregants from certain

of these hybrids was reported, most of which have terminal deletions of various regions of the long (q) arm of human chromosome 5 (7). The segregants were isolated by subjecting hybrids to a combination of selective pressures that required retention of the human *LARS* gene (previously referred to as *leuS*), which is located near the centromere on 5q, and loss of either the human *RPS14* (previously designated *emtB*) or *CHR* genes, both of which are located on the distal half of 5q (7). Segregants used in this study whose deletion breakpoints were characterized previously include HHW 207, HHW 208, HHW 209, HHW 212, HHW 213, and HHW 217 (7, 8). In addition, three new segregants, HHW 401, HHW 406,

and HHW 409, which were isolated in an analogous fashion, were examined. HHW 406 has lost both the human *RPS14* and *CHR* genes and has a terminal deletion of 5q, with the deletion endpoint in band q23. HHW 409 has lost the human *RPS14* gene, has retained the human *CHR* gene, and has an interstitial deletion that extends from band 5q23 to 5q34 (See Figs. 1 and 2). HHW 401 has lost the human *RPS14* gene function but does not have a detectable deletion.

HHW 207, HHW 208, HHW 213, HHW 406, and HHW 409 were derived from hybrid HHW 105, which contains human chromosome 5 exclusively (6, 7). HHW 209 and HHW 217 were isolated from hybrid

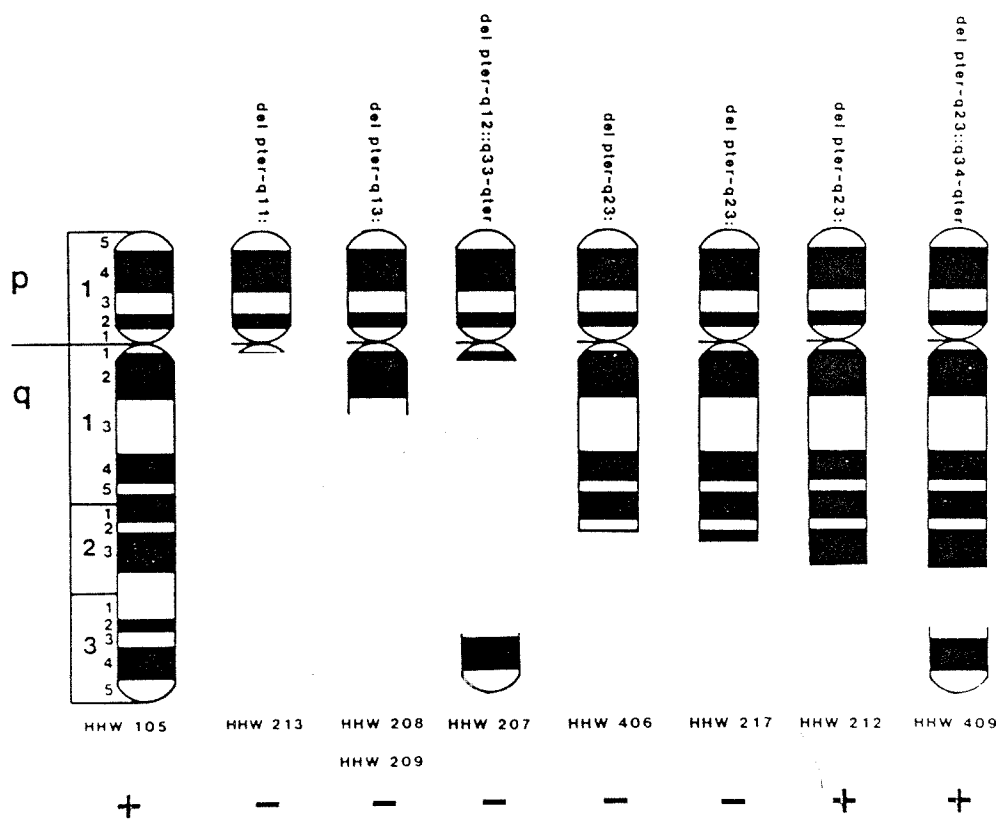


Fig. 1. Idiograms of the human chromosome 5 derivatives present in hybrid cell lines. The approximate deletion breakpoints are listed above each idiogram corresponding to the chromosome 5 derivative present in the different cell lines. The (+) or (-) at the bottom of the figure indicates the presence or absence of the human *DHFR* gene in each cell line.

HHW 212 HHW 217 HHW 406 HHW 105 HHW 409

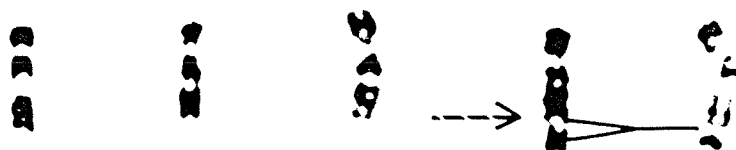


Fig. 2. Trypsin-Giemsa banded chromosome 5 derivatives present in various cell lines. The arrow on the left side of the normal human chromosome 5 present in HHW 105 indicates the approximate site of the terminal deletion endpoints in segregants HHW 212, HHW 217, and HHW 406. The chromosome 5 in HHW 212 appears to contain most, if not all, of band 5q23, while in both HHW 217 and HHW 406 the terminal deletion has removed the centromere distal one half to two thirds of band 5q23. The region of chromosome 5 that has been removed by the interstitial deletion in HHW 409 is indicated by the solid lines. It should be noted that the deletion endpoints in HHW 212 and HHW 217 were reversed in a figure in a previous report (7).

HHW 106, which contains human chromosome 3 and the Y chromosome in addition to chromosome 5. HHW 212 was derived from hybrid HHW 108, which contains only the human Y chromosome in addition to 5. Hybrid HHW 342 was derived from a different human parent than all the other cell lines and has a human chromosome 5 with a deletion extending from 5pter to 5p14 and human chromosome 3. The presence of chromosome 3 in HHW 209, HHW 217, and HHW 342 was determined by trypsin-Giemsa banding. The presence of the Y chromosome in HHW 209, HHW 212, and HHW 217 was determined by trypsin-Giemsa binding as well as by the presence of a Y chromosome-specific 4.3-kb EcoRI fragment which is detected by a cDNA probe (pAS-1) for the enzyme argininosuccinic acid synthetase (9; provided by Dr. A. L. Beaudet, Baylor College of Medicine).

Cytogenetic Procedures. Trypsin-Giemsa banding of metaphase chromosome preparations was performed as described by Worton and Duff (10).

Extraction of High-Molecular-Weight DNA. DNA was extracted from hybrid cell lines and segregants, which had been grown at 39°C, as described previously (11). Growth of

the cell lines at 39°C ensures that all viable cells contain the human chromosome 5 derivative (6, 7).

Preparation of Radioactive Probe. Nick-translation of the DHFR cDNA plasmid pHD84 (4) was carried out as described previously (5) to give a specific activity of $4-7 \times 10^8$ cpm/ μ g.

DNA Transfer and Hybridization Techniques. Samples of genomic DNAs (10 μ g) were digested to completion with an excess of EcoRI restriction enzyme, transferred to nitrocellulose filters by the method of Southern (12), and hybridized with the radioactive probe as described previously (5).

RESULTS

Organization of DHFR-Specific Sequences in Genomic DNAs from Human and CHO Cells. The plasmid pHD84 is a human DHFR cDNA clone containing the complete DHFR coding sequence (3, 4). Southern blots of EcoRI-digested human genomic DNA probed with pHD84 show a complex pattern consisting of fragments of the human *DHFR* gene which is amplified in the MTX-resistant cell line VA₂-B 6A3 (3) and of fragments containing other DHFR-specific sequences

(3, 5). In this paper, fragments that hybridize to the DHFR pHD84 probe, but that do not belong to the structural gene which is amplified in Va_2 -B6A3 cells, will be referred to as containing DHFR-related sequences.

Figure 3 (panel A, lane 1) illustrates the distribution of DHFR-specific sequences that hybridize to the pHD84 probe in *EcoRI* digests of genomic DNA from the human cell line HeLa S3. The fragments of the amplifiable structural gene are 13, 6, 4, 1.8 and 1.6 kb in size. These fragments, which span the entire reading frame for human DHFR, have been shown to be a part of the human struc-

tural gene for DHFR by molecular cloning and sequence analysis (13). Fragments not associated with the structural gene are designated in Fig. 3 by the letters a to g, and have molecular sizes of 18, 17, 3.8, 3.5, 2.3, 2, and ~1.6 kb, respectively (4, 5). The fragments d and e contain the intronless pseudogene Ψ HD1 (3), and the fragments b and c appear to be good candidates for containing the intronless pseudogene hDHFR- Ψ 2 and hDHFR- Ψ 1, respectively (14).

The hybridization pattern of an *EcoRI* digest of DNA from the CHO cell line UCW 56 probed with the human DHFR plasmid

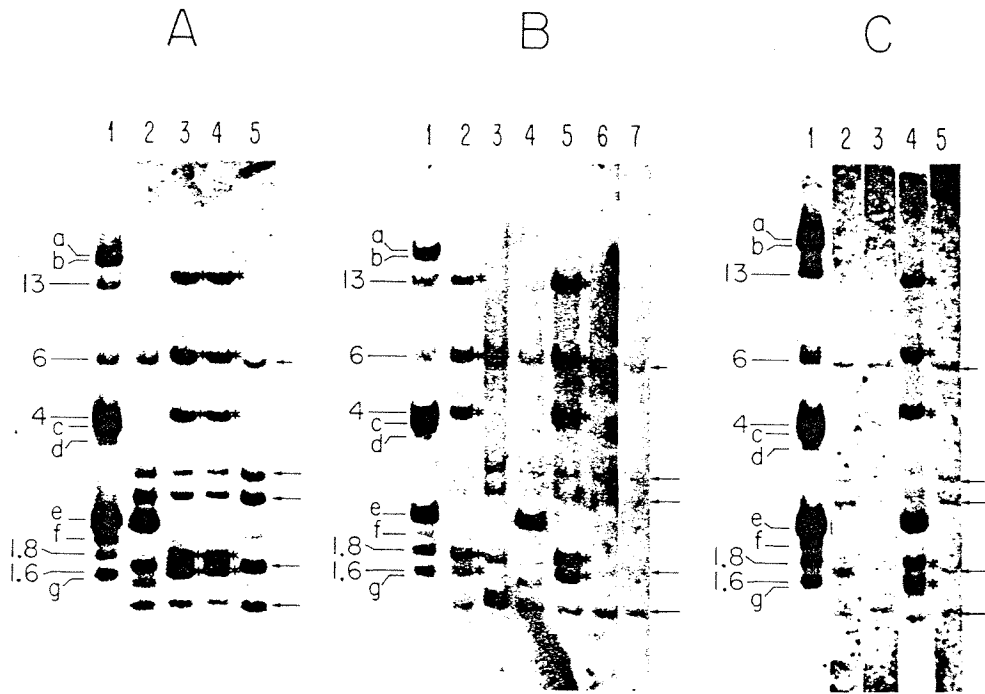


Fig. 3. Patterns of hybridization with a human DHFR probe of *EcoRI*-digested DNA from human-Chinese hamster ovary cell hybrid clones. DNA samples (10 μ g) were digested to completion with *EcoRI*, electrophoresed through agarose slab gels, transferred to nitrocellulose paper, and hybridized with the 32 P-labeled insert of pHD84. The numbers on the left of the panels indicate the normal human *DHFR* gene fragments and represent their sizes in kilobase pairs, while the letters refer to DNA fragments containing human DHFR-related sequences. The large arrows on the right of the panels indicate the Chinese hamster *DHFR* gene fragments; the small arrow refers to a DNA fragment probably containing a Chinese hamster DHFR-related sequence (see text). Panel A, lane 1 HeLa S3, human line; lane 2, HHW 217; lane 3, HHW 401; lane 4, HHW 409; lane 5, UCW 56, Chinese hamster ovary line. Panel B, lane 1, HeLa S3; lane 2, HHW 105; lane 3, HHW 213; lane 4, HHW 209; lane 5, HHW 212; lane 6, HHW 207; lane 7, UCW 56. Panel C, lane 1, HeLa S3; lane 2, HHW 406; lane 3, HHW 208; lane 4, HHW 342; lane 5, UCW 56. [The DNA sample from hybrid clone HHW 342 (lane 4) was run in a separate gel.]

pHD84 is shown in Fig. 3 (panel A, lane 5). Four of the seven EcoRI fragments containing the coding sequence of the CHO *DFHR* gene (15, 16), in particular those containing the coding regions I and part of II, III, IV, and V, can be seen (indicated by long arrows). The two small EcoRI fragments of ~0.25 kb and 0.8 kb are not visible, because they run out of the gel. Similarly, the predicted fragment of ~4.6 kb, containing the 3' end proximal coding segment present in exon VI of the exon VI of the CHO *DHFR* gene, is not seen, possibly due to the limited homology with the pHD84 probe (a faint band at the expected position was observed in blots with Chinese hamster of a different source). The 6-kb fragment probably contains a *DHFR*-related sequence; this may correspond to that previously reported not to be amplified in a CHO cell line containing amplified *DHFR* genes (17). The fragments of the human *DHFR* gene with sizes 13, 4, 1.8, and 1.6 kb migrate to positions in the gel free of CHO-specific bands. The 1.7-kb CHO *DHFR* gene fragment can be clearly seen to migrate between the 1.8- and 1.6-kb human fragments in Fig. 3 (panel A). Thus, it has been possible to score the human-CHO hybrid DNAs for the human *DHFR* gene using the 13-, 4-, 1.8-, and 1.6-kb bands as diagnostic of the entire gene. In addition, the EcoRI fragments a to g, containing *DHFR*-related sequences, are not obscured by cross-hybridizing CHO DNA fragments.

Analysis of Human DFHR Gene and Related Sequences in Human-CHO Cell Hybrid DNAs. Southern blots of EcoRI-digested DNA from the human-CHO cell hybrids analyzed here are shown in Fig. 3 (panels A-C). In all cell hybrid DNAs, the characteristic CHO *DHFR* and *DHFR*-related sequences can be seen. In contrast, the fragments of the human structural gene (indicated by asterisks) are present in only five of the 11 human-CHO DNA samples. In addition, in three of the samples, the fragments e and g can be seen. A surprising observation

was the absence of fragment d in the latter three samples, since fragments d and e together contain the entire pseudogene, Ψ HD1. From the relative intensities of band e in the EcoRI digests of DNA of HeLa S3 cells and of the above-mentioned hybrids, it can be concluded that the absence of band d is real and not due to inadequate sensitivity of detection. The results of the genomic blotting and chromosomal analysis for the cell hybrids investigated here are summarized in Table 1. The results are consistent with the location of the human *DHFR* gene in 5q23 and of the human pseudogene Ψ HD1 and *DHFR*-related fragment g in chromosome 3 (5 and present work).

DISCUSSION

Examination of Fig. 1 and Table 1 reveals the absence of the *DHFR* structural gene in segregants which contain chromosome 5 deletions including the major distal portion of 5q23 and, by contrast, the presence of the *DHFR* gene in segregants containing an intact 5q23. In particular, the absence of the *DHFR* gene in HHW 406 and HHW 217 and its presence in HHW 212 and HHW 409 indicate that the gene resides in 5q23.

The present data also provide information on the chromosomal location of the *DHFR*-related sequences, in particular of the human *DHFR* pseudogene Ψ HD1 (3). This has been shown to be an intronless pseudogene exhibiting a 92% sequence homology to the normal *DHFR* coding sequence (3). Fragment d has been shown to contain a nucleotide stretch corresponding to the 5' end proximal 91 nucleotides of the *DHFR* coding sequence, while fragment e contains a stretch corresponding to the remaining 473 nucleotides of the coding sequence (3). The presence of fragment e in cell hybrids carrying chromosome 3, specifically HHW 209, HHW 217, and HHW 342, together with the results of previous work (involving chromosomal segre-

Table 1. Occurrence of Human *DHFR* Structural Gene and Human *DHFR*-Related Sequences in Human-CHO Cell Hybrids Carrying Various Deletions in Human Chromosome 5

Hybrid	Chromosome 5 deletion	Other human chromosomes	Human <i>DHFR</i> gene	Human <i>DHFR</i> -related sequence
T05	none		+	
207	del pter-q12::q33-qter		—	
208	del pter-q13:		—	
209	del pter-q13:	3,Y	—	e,g
212	del pter-q23:	Y	+	
213	del pter-q11:	—	—	
217	del pter-q23:	3,Y	—	e,g
342	del :p14-qter	3	+	e,g
401	none detectable		+	
406	del pter-q23:		—	
409	del pter-q23::q34-qter		+	

gation analysis of murine-human somatic cell hybrids) (5), indicate that the human pseudogene Ψ HD1 is located in chromosome 3. However, the absence of fragment d in the *Eco*RI digests of DNA from the cell hybrids containing fragment e, which derived from two different human parents, is apparently in contrast with this assignment. The data appear to exclude a technical factor as an explanation for the absence of fragment d. A plausible interpretation of this finding is the existence of a polymorphism of this genomic region. Conceivably, this polymorphism could result in the presence of an extra *Eco*RI site within fragment d or in the absence from this fragment of the delimiting *Eco*RI site distal to the pseudogene: either of these situations could result in the displacement of band d to a position in the gel where it might not be detected (either as a result of size reduction or comigration with other bands). In this connection, the observation that the three samples exhibiting fragment e also exhibit fragment g, together with previously reported data on the most probable chromosome assignment of fragment g (5), suggests the possibility that fragments d and g result from the polymorphism being discussed here, with both g and d containing the 5' end proximal segment of Ψ HD1. Further work is needed to clarify this point.

The clear absence of fragment c from the three hybrids analyzed here which contain chromosome 3 would tend to exclude this chromosome as the site of the pseudogene h*DHFR*- Ψ 1, a possibility which had been suggested by earlier observations (5).

NOTE ADDED IN PROOF

In a recent paper (Funanage, V.L., Myoda, T.T., Moses, P.A., and Cowell, H.R., *Mol. Cell. Biol.* 4: 2010–2016, 1984), the assignment of the human *DHFR* gene to the q11–q22 region of chromosome 5 has been reported.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Schimke, R.T. (1982). In *Gene Amplification*, (ed.) Schimke, R.T. (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), pp. 317–333.

2. Masters, J., Keeley, B., Gay, H., and Attardi, G. (1982). *Mol. Cell. Biol.* **2**:498-507.
3. Masters, J.N., Yang, J.K., Cellini, A., and Attardi, G. (1983). *J. Mol. Biol.* **167**:23-36.
4. Morandi, C., Masters, J.N., Mottes, M., and Attardi, G. (1982). *J. Mol. Biol.* **156**:583-607.
5. Maurer, B.J., Barker, P.E., Masters, J.N., Ruddle, F.H., and Attardi, G. (1984). *Proc. Natl. Acad. Sci. U.S.A.* **81**:1484-1488.
6. Dana, S., and Wasmuth, J.J. (1982). *Somat. Cell. Genet.* **8**:245-264.
7. Dana, S., and Wasmuth, J.J. (1982). *Mol. Cell. Biol.* **2**:1220-1228.
8. Groffen, J., Heisterkamp, N., Spurr, N., Dana, S., Wasmuth, J.J., and Stephenson, J.R. (1983). *Nucleic Acids Res.* **11**:6331-6339.
9. Daiger, S.P., Wildin, R.S., and Su, T.-S. (1982). *Nature* **298**:682-684.
10. Worton, R., and Duff, C. (1979). *Methods Enzymol.* **53**:322-344.
11. Pellicer, A., Wigler, M., Axel, R., and Silverstein, S. (1978). *Cell* **14**:133-141.
12. Southern, E.M. (1975). *J. Mol. Biol.* **98**:503-517.
13. Yang, J.K., Masters, J.N., and Attardi, G. (1984). *J. Mol. Biol.* **176**:169-187.
14. Chen, M.-J., Shimada, T., Davis Moulton, A., Harrison, M., and Nienhuis, A.W. (1982). *Proc. Natl. Acad. Sci. U.S.A.* **79**:7435-7439.
15. Milbrandt, J.D., Azizkhan, J.C., Greisen, K.S., and Hamlin, J.L. (1983). *Mol. Cell. Biol.* **3**:1266-1273.
16. Carothers, A.M., Urlaub, G., Ellis, N., and Chasin, L.A. (1983). *Nucleic Acids Res.* **11**:1997-2012.
17. Graf, L.H., Jr., and Chasin, L.A. (1982). *Mol. Cell. Biol.* **2**:93-96.

CHAPTER TWO ADDENDUM

Figure 2-1. In situ hybridization of human DHFR gene cDNA to normal human lymphocyte metaphase spreads.

Figure Legends

Figure 2-1. In situ hybridization of human DHFR gene cDNA to normal human lymphocyte metaphase spreads.

Methods

Human male metaphase spreads were prepared from MTX-synchronized peripheral blood lymphocyte cultures. Slides were hybridized (see Methods, Chapter Three) to pHD84, a plasmid containing a complete human DHFR gene cDNA insert. Results are presented as the recorded distribution of grains observed with the following exception: due to the difficulty in distinguishing chromosomes 4 and 5 when grains were over the long arm of the chromosome, the raw grain counts for chromosome 4 and 5 were recorded together. The number of grains expected for chromosome 4 assuming all grains were randomly distributed was calculated as described in Methods, Chapter Three, and this number was subtracted from the summed chromosome 4 and 5 grain count. The result is a uniform grain distribution on chromosome 4 representing the random background count expected for a chromosome of its size and the remaining grains on chromosome 5 attributable to the DHFR gene locus.

Discussion

The result of in situ hybridization on normal human lymphocytes performed after deletion mapping results suggest that the DHFR gene locus is located closer to the centromere (approximately 5q15) than the 5q23 assignment indicated by deletion mapping (Chapter Two). The cause of the discrepancy is unclear. However, both methods indicate the locus resides in the 5q arm. This new

assignment agrees with the 5q11-5q22 location reported in HGM 10 (134), (NOTE: assignment as represented in figure of HGM 10 is in error. References listed in HGM 10 indicate 5q11-5q22 is the correct assignment). Further, results suggest that the DHFR pseudogene, Psi-HD1 (hDHFR-Psi-4 (73)), assigned to chromosome 3 may reside at about 3p24 and that another pseudogene (see Chapter One, hDHFR-Psi-2) may reside at 6p21.

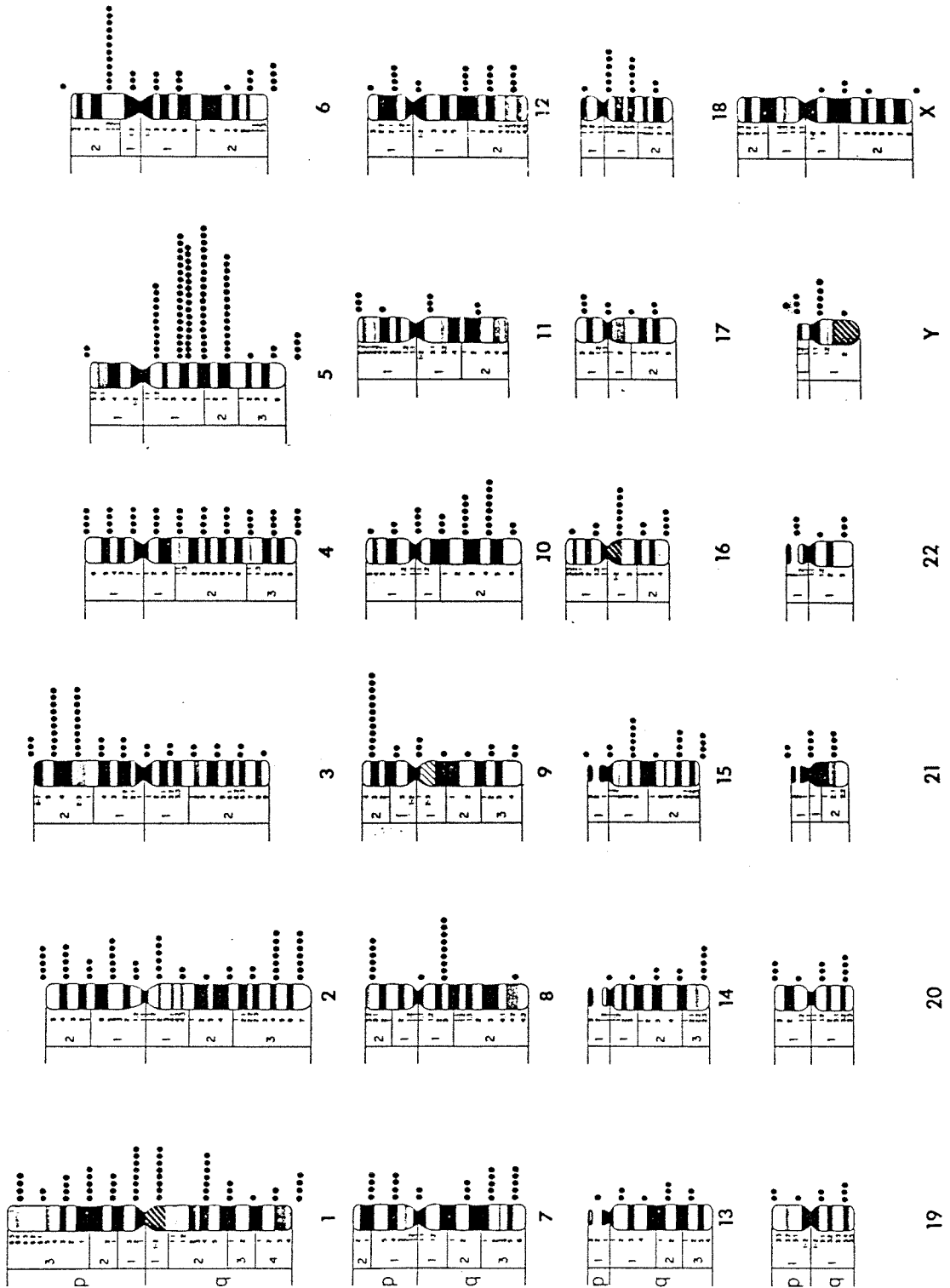


Figure 2-1

CHAPTER THREE

Dihydrofolate Reductase Gene Amplification in the Human Cell Line VA₂-6A3

DIHYDROFOLATE REDUCTASE
GENE AMPLIFICATION
IN THE HUMAN CELL LINE VA₂-6A3

INTRODUCTION

Selective gene amplification has been observed in drug-resistant mammalian cell lines and in tumors or cell lines derived from them (3) and is generally accompanied by optically visible chromosomal abnormalities. The abnormalities include minute chromosomes (DMs) (acentromeric, self-replicating, extrachromosomal elements), chromosomes containing an expanded "homogeneously staining region" (HSR) and small chromatin bodies (SCB) (6-9). Both minute and HSR chromosomes have been shown to contain the amplified genes responsible for resistance in drug-selected cell lines (3-8). HSRs have also been shown to contain amplified oncogene sequences in human tumor cell lines (28-32). The generative mechanism(s) of gene amplification and attendant chromosomal alterations in mammalian cells is not known. Suggested models to explain the phenomenon include unequal sister chromatid exchange (51,52), chromosomal fragmentation (6), rolling-circle replication (57), onion-skin reinitiation (53,54) and yeast 2-micron element-like overreplication (55). The relationship, if any, between minute and HSR chromosomes is also not clear.

Of possible relevance to the generative mechanism(s) of gene amplification is the reported predominance of one form of chromosomal abnormality over another in different cell lines of the same species, with some

indications also of species specificity (3,6). In general, murine cell lines contain either DMs or HSRs, while hamster lines contain HSRs preferentially. Only the occasional occurrence of both abnormalities in the same cell have been described (77,96). The question has remained whether this species-specific bias is a true reflection of differential cellular mechanisms or results from the paucity of analyzed time points in the stepwise evolution of the resistant phenotypes.

In order to investigate this question, and to gain insight into the generative mechanism(s) of mammalian gene amplification in human cells, a detailed study of the evolution of a human cell line, VA₂-6A3, was performed. VA₂-6A3 is one of a series of human cell variants isolated in this laboratory (13) that is resistant to 1.8×10^{-4} M D,L-methotrexate (MTX). These cell lines exhibit variability in phenotypic and chromosomal constitution, including variability in the number of DMs and HSRs (13). The evolution of VA₂-6A3 has been analyzed with respect to dihydrofolate reductase (DHFR) protein level, gene copy number and genomic location, chromosomal constitution and growth rate.

MATERIALS AND METHODS

Materials. MTX used as a selective agent for the isolation of DHFR gene-amplified variants was obtained from Sigma Chemical Co. as \pm Amethopterin.

Cell Line and Method of Growth. The isolation of VA₂-6A3 from the human cell line VA₂-B, an azaguanine-resistant subclone of the simian virus

40 (SV40) – transformed line WI-18-VA₂ has been reported previously (13). Briefly, VA₂-6A3, one of several variants resistant to 1.8×10^{-4} M D,L- MTX, was isolated by stepwise selection and propagated for a number of months after reaching the final MTX concentration. Samples were frozen at stages during stepwise selection and subsequent propagation and analyzed after thawing.

Chromosome Analysis. Cells grown on plates overnight were arrested by the addition of colchicine or colcemid for 20–40 min., trypsinized, swollen in hypotonic solution 15–45 min. at 37°C and fixed with methanol:acetic acid (3:1). Metaphase spreads were stained with orcein or banded by the trypsin-Giemsa method, photographed with Kodak Techpan 2415 film and printed onto Kodak Ektamatic SC paper for analysis. With banded spreads, two exposures of each spread were printed and analyzed; one emphasizing banding, the other overexposed to demonstrate minute chromosomes. Spreads were analyzed for chromosome number, minute chromosome number and for the presence of marker chromosomes. In cells with HSRs, the total HSR length was expressed as a fraction of the length of chromosome 1 as measured in the same spread (1 unit = length of chromosome 1 in spread). As an exception, the length of the DHFR gene containing region of HSR5 was estimated in a different way, as described in the text below.

Cell Protein Extraction and Analysis. Cells were grown on plates overnight, trypsinized, washed three times in isotonic saline and resuspended in 0.15 M KCl, 0.01 M Tris pH 7.5, 0.003 M mercaptoethanol. The cells were sonicated to disruption and centrifuged for one hour in a SW50.1 rotor at 18K

rpm at 2°C. A Lowry protein assay was performed on the supernatant using bovine serum albumin as a standard and 15 µg samples were electrophoresed through 10% sodium dodecyl sulfate (SDS)- polyacrylamide gels.

DHFR Gene Copy Number Estimation. High molecular weight cellular DNA isolated by the method of Chambon (70), plasmid DNA and *E. coli* DNA were quantitated fluorometrically. Standards corresponding to 0.5 to 200 copies of DHFR gene per haploid genome (3.3×10^9 bp) were made by adding the human DHFR cDNA-containing plasmid pHD84 (71) to 2 µg of *E. coli* DNA (13 samples). The standards and 2 µg samples of genomic DNAs were transferred onto a nitrocellulose membrane using a dot blot apparatus. The membrane was incubated with the ^{32}P -labeled DHFR cDNA insert from pHD84 at 68°C for 20 hours in a hybridization mixture consisting of 0.1 % sodium pyrophosphate, 0.1 % NaDodSO₄, 10x Denhardt's solution (1 x Denhardt's solution = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 6x NaCl/Cit (1x NaCl/ Cit = 0.15 M NaCl/0.015 M sodium citrate), 100 µg sonicated salmon sperm DNA, 10% dextran sulfate, washed for 30 min. in hybridization mixture without dextran sulfate at 68°C, followed by two washes, for 30 min. each, in 2x NaCl/Cit, 0.1 % NaDodSO₄ at 68°C, and two washes, for 30 min. each, in 2x NaCl/Cit, 0.1% NaDodSO₄ at 68°C, and three washes, for 20 min. each, in 0.5x NaCl/Cit, 0.1 % NaDodSO₄ at 68°C. After autoradiography, the regions of the membrane containing the samples were excised with a hole punch and the ^{32}P cpm measured in a scintillation counter. Standard curves of gene copy number versus ^{32}P cpm were made using a linear regression program in two ranges: (0.5–50 copies/haploid genome,

correlation coefficient = .986) and (0.5–200 copies/ haploid genome, correlation coefficient = .989). The gene copy number per haploid genome for each stage derived from these curves was then corrected for the estimated amount of DNA per cell as follows: The lengths of the chromosomes in a banded metaphase spread representative of that stage were measured as a fraction of the average lengths of the chromosome 1 in that spread. Chromosome lengths were summed and the total compared to that expected for a normal human diploid cell (approximately 25 chromosome 1 unit-lengths per cell). One to five metaphase spreads per stage were analyzed (depending on the variability exhibited by the cell population at that stage). A constant chromatin packing density is assumed. Correction was also made for the percentage of cells that exhibited pseudotetraploidy at each stage. The DNA content per cell estimated in this way was then used to correct the gene copy number estimates based on normal haploid genome sizes. The result estimates the average DHFR gene copy number per cell at a given stage. Correction for cross hybridization of known DHFR pseudogenes (72,73) was not made as Southern blots of restriction endonuclease digested DNA probed with DHFR cDNA demonstrated that none were amplified at any cell stage. DHFR pseudogenes therefore only interfere with gene copy estimates at low gene copy number. An estimate of the DHFR gene content of Hela Bu25 (approximately 8.4×10^9 bp per cell), a non-amplified human cell line, by this method gives about 15 DHFR-gene related copies/cell (normal gene plus pseudogene copies), which compares favorably with an expected estimate of about 12 copies/cell (one normal plus about five pseudogene copies per haploid genome).

In situ Hybridization. In situ hybridization to metaphase spreads was made after the method of Harper (74) using either the DHFR cDNA insert of pH84 or the whole plasmid labeled with either ^{125}I or ^3H . The slides to be incubated with the ^{125}I -labeled probe were washed in a solution made of 0.5 ml acetic anhydride in 200 ml 0.1 M triethanolamine, pH 8.0, for ten minutes, then dehydrated in an ethanol series prior to denaturation to decrease nonspecific background hybridization. The slides were coated with either Kodak NTB-2 or NTB-3 photographic emulsion and banded with Wright stain after development. The metaphase spreads were photographed and printed for analysis. Chromosome identification was based on banding as well as relative chromosome lengths and centromere position. As the distinction between chromosome 4 and chromosome 5 was not easily made, particularly when grains were present over the long arm of the chromosome, grain counts from chromosome 4 and 5 were scored together. The data were plotted after calculating the number of grains per unit-length chromatin expected if all grains recorded were randomly distributed (length of chromosome 1 = 1 unit-length chromatin), multiplying this number by the average copy number per metaphase spread of a given chromosome and its unit length, and then subtracting this value from the actual number of grains observed over the chromosome. Ex: total grains observed on all spreads = 569, avg. number of unit-length chromatin per spread = 29, then expect 19.6 grains per unit-length chromatin. For chromosome 6, (9 grains observed) – (avg. 2 copy of chromosome 6/spread) (0.7 unit-length chromatin/chromosome 6) (19.6 grains/unit-length chromatin) = -18 grains. By this method, large positive values represent significant values above background.

RESULTS

DHFR protein levels during stepwise selection and propagation. VA₂-6A3 cells were exposed to increasing concentrations of MTX over a period of eleven months, followed by propagation in 1.8×10^{-4} M D,L-MTX for an additional nine months. The increasing MTX resistance was correlated with an increasing quantity of DHFR protein per cell. When equal amounts of protein were run on sodium dodecyl sulfate-polyacrylamide gels, a band corresponding to a polypeptide with a M_r of about 22,000, previously shown to be DHFR (13), increased in intensity concurrently with the increase in MTX concentration up to 11 months, and thereafter increased slightly up to 14 months, even though the MTX level had remained constant after 11 months (Figure 3-1).

DHFR gene copy number. Estimates of the DHFR gene copy number per cell at different stages over a twenty month period are shown (Figure 3-2). The parental line VA₂-B, has a moderately increased DHFR gene copy number (72), although there is no evidence of previous exposure to MTX and exhibits only a slight increase in DHFR activity (13). The initial selection step resulted in a drop of the gene copy number from approximately 60 copies in VA₂-B to that expected for an unamplified hyperdiploid cell (see Methods). Subsequently, the gene copy number increased with time, even after the MTX levels had ceased to increase. This increase in gene copy number was associated with a decrease in doubling time, from about 62 hours at Month 8

(1.6×10^{-4} M D, L- MTX) to about 30 hours at Month 19 (1.8×10^{-4} M D,LMTX) (data not shown), and with karyotypic changes (see below).

Chromosomal constitution. The karyotype of the parental line, VA₂-B, is shown in Figure 3-3. While the parent of VA₂-B, WI-18-VA₂, is reported to be a SV40-transformed lung carcinoma cell line, VA₂-B exhibits several marker chromosomes associated with Hela cell lines including the chromosomal rearrangements 3q:1q, 3p:5q, M7 and M8. Further, neither VA₂-B or VA₂-6A3 currently demonstrate SV40 DNA sequences as judged by Southern blot analysis (data not shown). While convergent evolution of karyotype cannot be excluded, the derivation of VA₂-B must remain uncertain. (In this laboratory, VA₂-B has exhibited its current karyotype in a stable fashion for a number of years. The VA₂-B karyotype is also distinct from other Hela derivatives maintained in the laboratory.)

Exposure to MTX resulted in the appearance of chromosome fragments (in early stages), chromosomes containing an HSR and the appearance, then elimination, of minute chromosomes (Figures 3-2,-4,-5 and Table 3-1). Figure 3-2, shows the changes with time in DHFR gene copy number, number of HSRs and number of minute chromosomes during selection and maintenance in MTX of VA₂B-6A3. Of particular interest was the formation of chromosomes shown to contain amplified DHFR genes prior to the development of visible minute chromosomes. In particular, three chromosomes designated HSR2 (11p+), HSR4 and HSR5 were shown by in situ hybridization to contain DHFR genes within the HSR (or abnormally staining region (ABR) in HSR5) portions of their chromosomes at Month 4 prior to the development of numerous minute

chromosomes by Month 9 (see below). It is also of interest that HSR4 and HSR5 were eliminated from the population by Month 9 although HSR2 was retained. After continued growth in 1.8×10^{-4} M D,L- MTX, the chromosome constitution continued to change with the appearance of HSR1 at Month 14 and HSR3 at Month 19 (accompanied by a change to pseudotetraploidy in most cells). Acquisition of these chromosomes and increase in median chromosome number was associated with a decline in the number of minute chromosomes and their eventual elimination from the population by Month 19.

Localization of the DHFR genes. Figure 3-6 shows a typical metaphase spread after in situ hybridization using a radiolabeled human DHFR cDNA probe. In situ hybridization results for all stages analyzed are summarized in Figures 3-7a-d. In situ hybridization failed to localize the increased DHFR gene copies in the VA₂-B parental line. While chromosomes 3, 11 and 12 exhibited greater than expected grain counts (see Methods and Figure 3-7a), analysis of the distribution of the grains along the lengths of these chromosomes failed to demonstrate convincing localization (data not shown). This unexpected result may indicate that the amplified gene copies in VA₂-B exist extrachromosomally (see Chapter Seven). In contrast, DHFR sequences were convincingly demonstrated on HSR chromosomes that appeared during the course of selection (Figure 7b-d). The data suggests that not all DHFR gene-containing regions contain the same number of genes per unit-length chromatin. This is readily apparent when HSR2 and HSR5 at Month 3 are compared (Figure 3-7b). Although HSR2 and HSR5 occur with approximately equal frequencies in the population at this stage, HSR5 exhibited more grains

localized to a smaller region than HSR2. Similarly, although HSR4 appears in a minority of cells at this stage, the total number of grains observed was about the same as HSR2. This implies that both HSR4 and HSR5 contain DHFR sequences at a higher density per unit length chromatin than does HSR2, suggesting the possible independent origin of these HSRs. In contrast, HSR1, HSR2 and HSR3, (Figure 3-7d) exhibit roughly the number of grains expected based on the size of their respective HSR regions and chromosome frequency, assuming the same gene density per unit-length chromatin. This may imply that HSR1 and HSR3 were formed from rearrangement or translocation of HSR2.

DISCUSSION

The primary purpose of this work has been to investigate the evolution of the MTX-resistant human cell line VA₂B-6A3 with respect to the time of appearance and possible relationship of the DMs and HSRs. The demonstration that both types of chromosomal abnormalities can coexist within the same cell as the majority cell type of a population for extended periods of time during its evolution is a novel observation, and may reflect a basic difference in the mechanism(s) producing gene amplification in human cells or a paucity of data on the evolution of rodent cell lines, with coexistence of the two types of chromosome abnormalities actually occurring at some stage. While the existence of DHFR genes in minute chromosomes has not been directly demonstrated in this study, their occurrence in these structures is strongly supported by previous work (3,6) and, in the present work, by the parallel increase in minute chromosomes and gene copy number in

concurrence with a constant or decreasing number of genes contained in HSRs copies (Months 3–11) and from the decrease in minute chromosomes paralleling the increases in genes contained in HSRs (Months 11–19) (Figure 3-2). In this study, minute chromosomes appeared in large number after HSR-bearing chromosomes (see Legend, Figure 3-2) and were selected for under conditions of increasing selective pressure (Months 3–9). Upon stabilization of selection, cells containing HSRs demonstrated a growth advantage (Months 9–19). This observation is presumably a reflection of the ability of acentromeric minute chromosomes, once formed, to more rapidly produce an increase in gene copy number, under conditions of increasing selective pressure, by asymmetric segregation at mitosis, in contrast to the greater mitotic stability afforded by genes contained in HSRs when under stable selective pressure.

It is also notable that the actual evolution of an HSR was not observed during this study: while several HSRs were formed, all appeared in their final form without intermediate steps (in the size of the HSR region itself). This "catastrophic" appearance may imply that the generative mechanism of HSRs operates over very short periods of time, perhaps as short as a single cell generation (see Chapter Six). Furthermore, the existence of HSRs with apparently different gene densities per unit chromatin (HSR2, HSR4, HSR5) may imply that generative events can occur independently and repeatedly.

It was also observed that, in contrast to reports in other species, no HSR formed at the site of the normal DHFR gene (5q11-5q23), although chromosome 5 may have been involved in the formation of HSR5 as suggested by its Giemsa banding appearance (Figure 3-4). This may imply that

either translocation or extensive chromosomal rearrangement may be an obligatory step in the amplification process of this locus in human cells. A similar suggestion has been made regarding the amplification of *c-myc* in neuroblastoma cell lines (30). The similarities in appearance and gene density per unit chromatin of HSR1, HSR2 and HSR3 may imply that new HSR chromosomes (HSR1, HSR3) may be formed from preexisting ones (HSR2) by chromosomal rearrangement.

The inability to localize the approximately 60 extra DHFR gene copies in the VA₂-B parental line is curious and may indicate a stable, extrachromosomal location (see Chapter Seven). The only slight increase in DHFR activity in VA₂-B (13,71) and the loss of the extra genes upon the initial selection step (Figure 3-2) would imply that the excess of DHFR mRNA transcribed from these genes is not efficiently translated, as the expression of even a fraction of 60 DHFR genes would be expected to allow for adequate growth in the MTX concentration used at initial selection. Why these presumably inactive genes would be lost upon selection is not clear, but the observed increase in fragmented chromosomes and decrease in mean chromosome number at early selection stages suggests the possibility that fragile sites induced in chromatin by inhibitors of purine metabolism (75) may have functioned to destabilize the chromatin regions containing them, or that growth advantage exists for cells that lose nonessential DNA.

The current study has unfortunately not been able to distinguish whether the formation of HSRs and the formation of minute chromosomes are mechanistically separate phenomena or reflect two phases of the same process. Although HSR chromosomes were observed before bona fide minute

chromosomes in this study (see Legend, Figure 3-2), the recent reports that amplified genes may reside in submicroscopic, extrachromosomal structures (Chapter Seven, 76) means that the pre-HSR existence of such structures cannot be excluded. (Alternatively, these submicroscopic structures may represent a molecular intermediate of both HSRs and minutes, or yet a third unrelated mechanism for gene amplification.) It is possible that these questions may be addressed by more frequent karyotypic observations at early selection stages (perhaps as frequently as every other generation) coupled to an evaluation of the presence of submicroscopic structures.

<u>[MTX]/Month</u>	<u>Chromosome Number (%)</u>	<u>Minute average Number (Range)</u>	<u>Marker Chromosome (%)</u>
5×10^{-8} M/1 (N=116) ⁺	1S= 61 ± 2 (95) 2S= 116 ± 4 (4) 2.5S= 152 (1)	1.7 ± 5 (0-46) 0.4 ± 0.9 (0-2) 0	chromosomal fragments
3×10^{-6} M/4 (N=56)	1S= 53 ± 2 (84) 1.5S= 76,85 (4) 2S= 105 ± 4 (11) 4S= 190 (2)	0.3 ± 1.3 (0-8) 0,0 0, (one spread=47) 0	HSR2(2), HSR5(4), HSRs4+5(7), HSRs2+4+5(2), HSRs2+5(84)
1.6×10^{-4} M/9 (N=35*)	1S= 56 ± 2 (97) 2S= 109 (3)	131 ± 66 (46-388) 100	HSR2(100)
1.8×10^{-4} M/11 (N=28)	1S= 56 ± 1 (96) 2S= 113 (4)	136 ± 74 (42-322) 263	HSR2(100)
1.8×10^{-4} M/14 (N=30)	1S= 54 ± 3 (57) 1S= 54 ± 2 (23) 2S= 105 ± 7 (20)	115 ± 65 (5-228) 8 ± 6 (0-17) 245 ± 200 (80-563)	HSR2(100); HSRs1+2(100); HSR2(100)
1.8×10^{-4} M/16 (N=34)	1S= 55 ± 6 (65) 1S= 55 ± 2 (24) 1.5S= 79,84 (6) 2S= 103,104 (6)	36 ± 37 (4-171) 5 ± 6 (0-15) 3,171 106,25	HSR2(100); HSRs1+2(100); HSR2(100); HSR2(100)
1.8×10^{-4} M/20 (N=47*)	1S= 54 ± 2 (26) 1.5S= .85,83 (4) 2S= 107 ± 5 (68) 4S= 217 (2)	16 ± 12 (0-33) 0,10 8 ± 7 (0-24) 8	HSR1(8), HSRs2+3(8), HSRs1+2+3(8), HSRs1+2(75); HSRs1+2(50), HSRs1+2+3(50); HSRs1+2(9), HSRs1+2+3(91); HSRs1+2+3(100);

*Doubling time: Month 9 = 62 hours
Month 20 = 30 hours
+Number of metaphases analyzed

Table 3-1. Chromosomal changes in cell line
VA₂-6A3 during MTX selection

Figure Legends

Figure 3-1. Electrophoretic patterns in sodium dodecyl sulfate-polyacrylamide gel of cell protein extracts from the cell line VA₂-6A3 during MTX selection. Arrow indicates DHFR protein. Lanes: a, VA₂-B parental; b, Month 1 (5×10^{-8} M MTX); c, Month 4 (3×10^{-6} M MTX); d, Month 9 (1.6×10^{-4} M MTX); e, Month 11 (1.8×10^{-4} M MTX); f, Month 14 (1.8×10^{-4} M MTX); g, Month 16 (1.8×10^{-4} M MTX); h, Month 20 (1.8×10^{-8} M MTX).

Figure 3-2. Average DHFR gene copy per cell, DM per cell and HSR units per cell in cell line VA₂-6A3 during selection. The HSR units were recorded as described in Methods, except at Month 4, where the gene contribution of HSR5 was estimated from in situ hybridization data (Figure 3-7b) and translated into an equivalent chromatin length through comparison with HSR2. One HSR unit = chromatin equal to length of chromosome 1 in metaphase spread. DM were recorded as single minute chromosomes. The presence of very small DMs in early stages cannot be excluded (see Method, Chapter Four).

Figure 3-3. Representative karyotype of VA₂-B parental line.

Figure 3-4. Representative metaphase spreads from cell line VA₂-6A3 during MTX selection. a, Month 4; b, Month 11; c, Month 20. Note: The sample from Month 4 may contain a few very small DMs; pseudotetraploidy of Month 20. Arrows indicate HSR chromosomes.

Figure 3-5. HSR-bearing marker chromosomes observed in cell line VA₂-6A3

throughout MTX selection. Bar equals equivalent length of chromosome 1. HSR2 resembles a 11p⁺; HSR4 resembles a 1p⁺. The bracket on HSR5 indicates the region that hybridizes with a DHFR cDNA probe (see Figures 3-6 and 3-7b). The appearance of the p arm and proximal q arm of HSR5 suggests that this chromatin may derive from chromosome 5.

Figure 3-6. Representative in situ hybridization to a metaphase spread during MTX selection (Month 4) using human DHFR cDNA. Demonstrated is hybridization to HSR2 and HSR5. Inset are similar chromosomes from another metaphase spread.

Figure 3-7a-d. Results of in situ hybridization to metaphase spreads from cell line VA₂-6A3 throughout MTX selection using human DHFR cDNA. a, VA₂-B parental, 21 spreads; b, Month 4, 48 spreads; c, Month 16, 67 spreads; d, Month 20, 40 spreads. In Figure 3-7a, the grains over chromosome 11 of VA₂-B do not localize to a given region (data not shown). In Figure 3-7b-d, the percentage of grains over chromosome localized to region indicated.

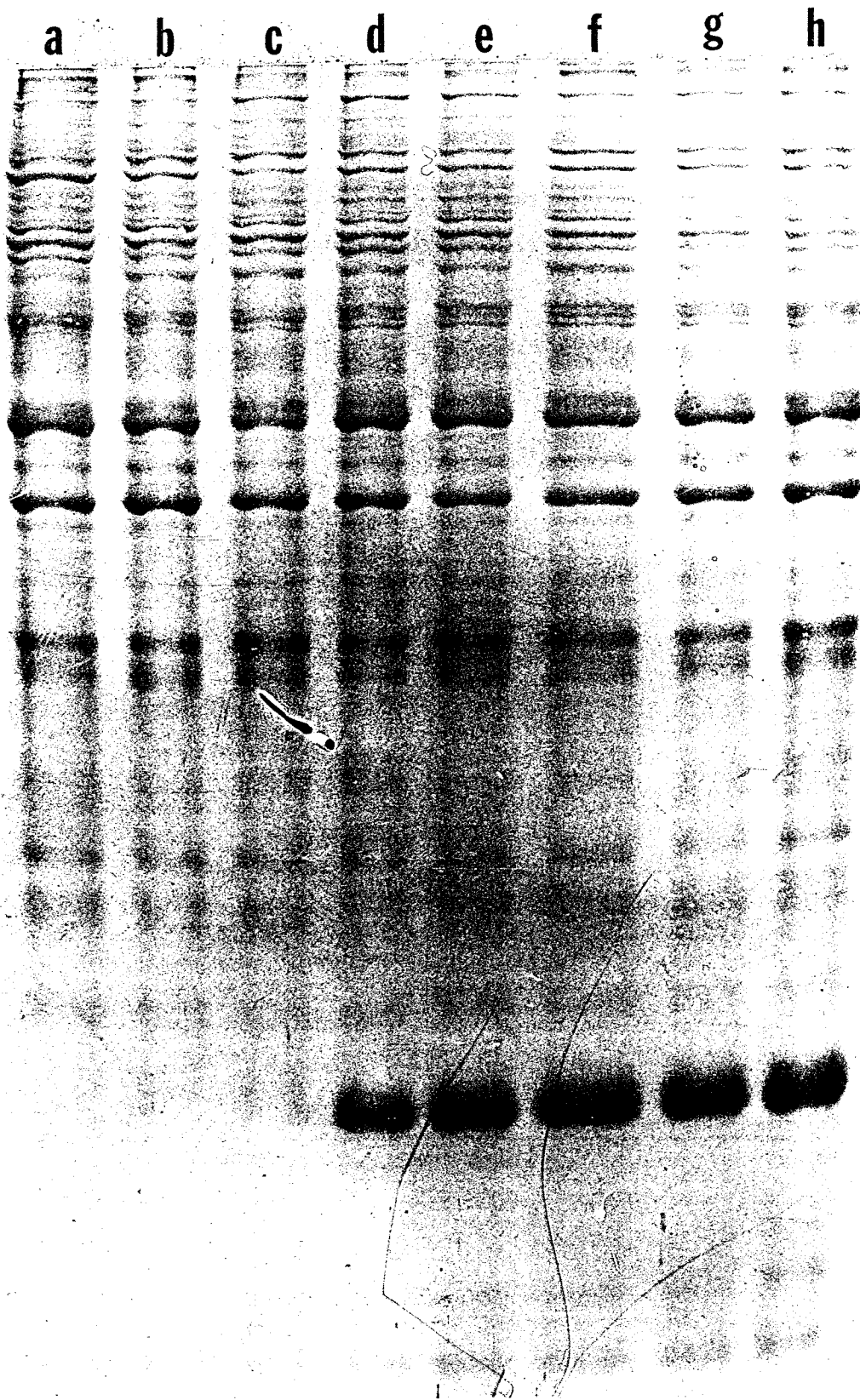


Figure 3-1

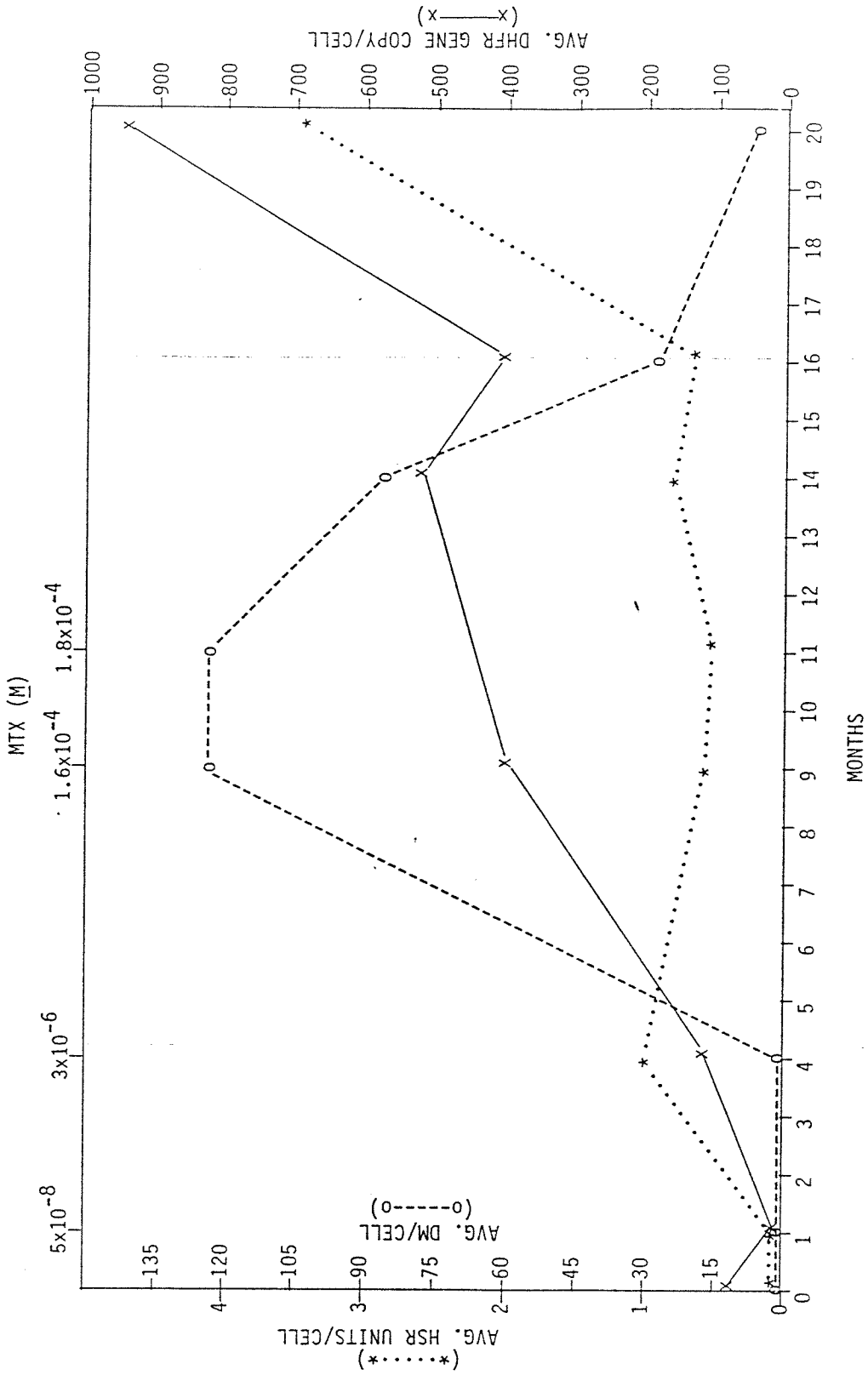


Figure 3-2



Figure 3-3

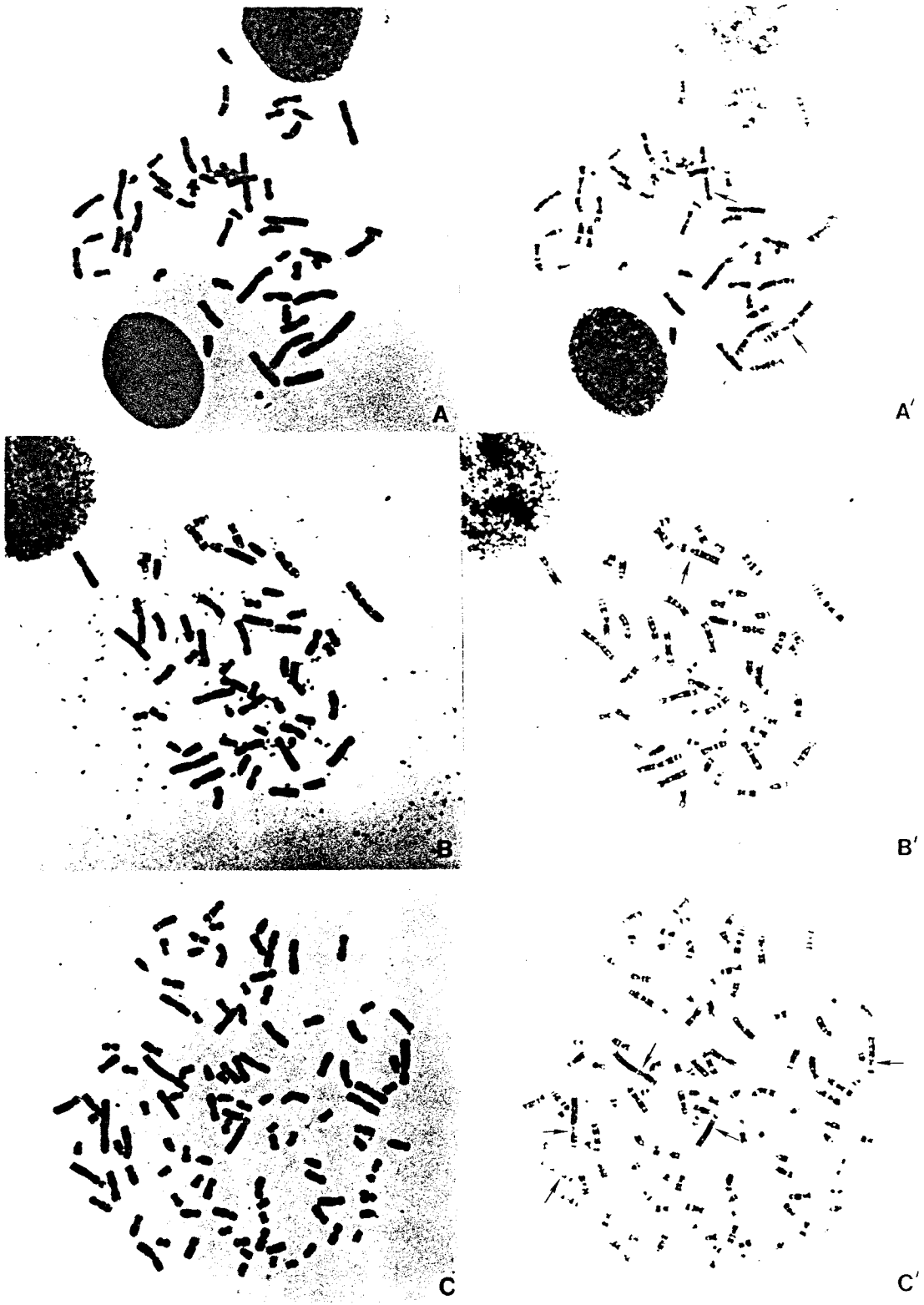


Figure 3-4

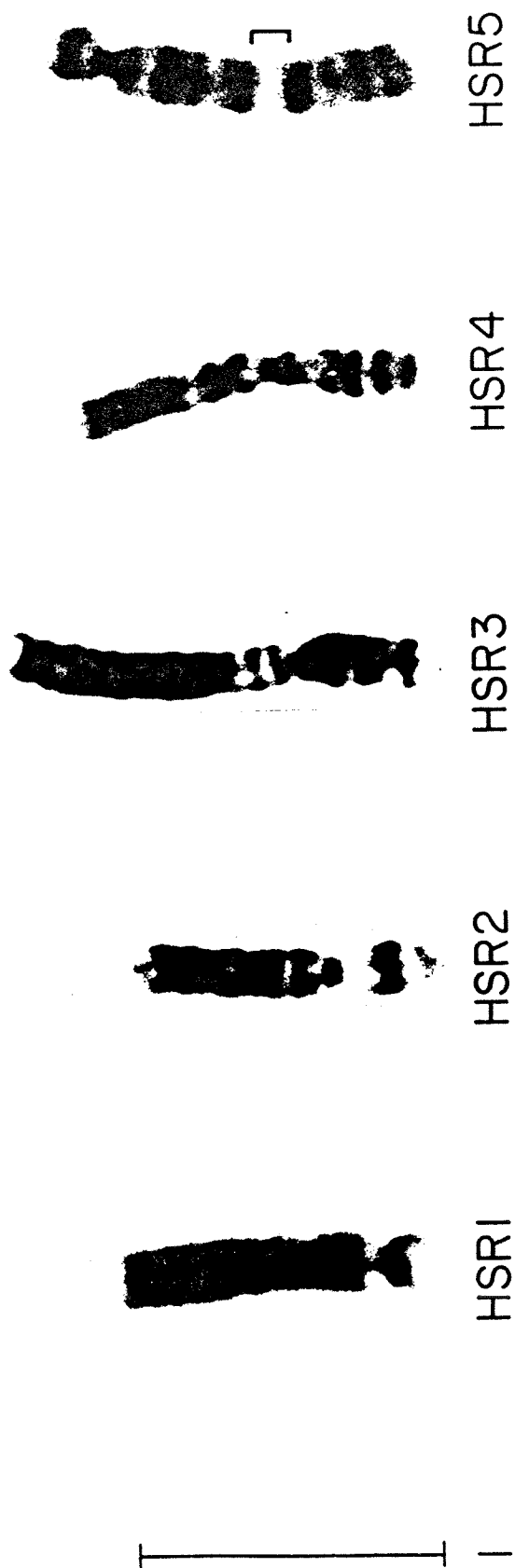


Figure 3-5



Figure 3-6

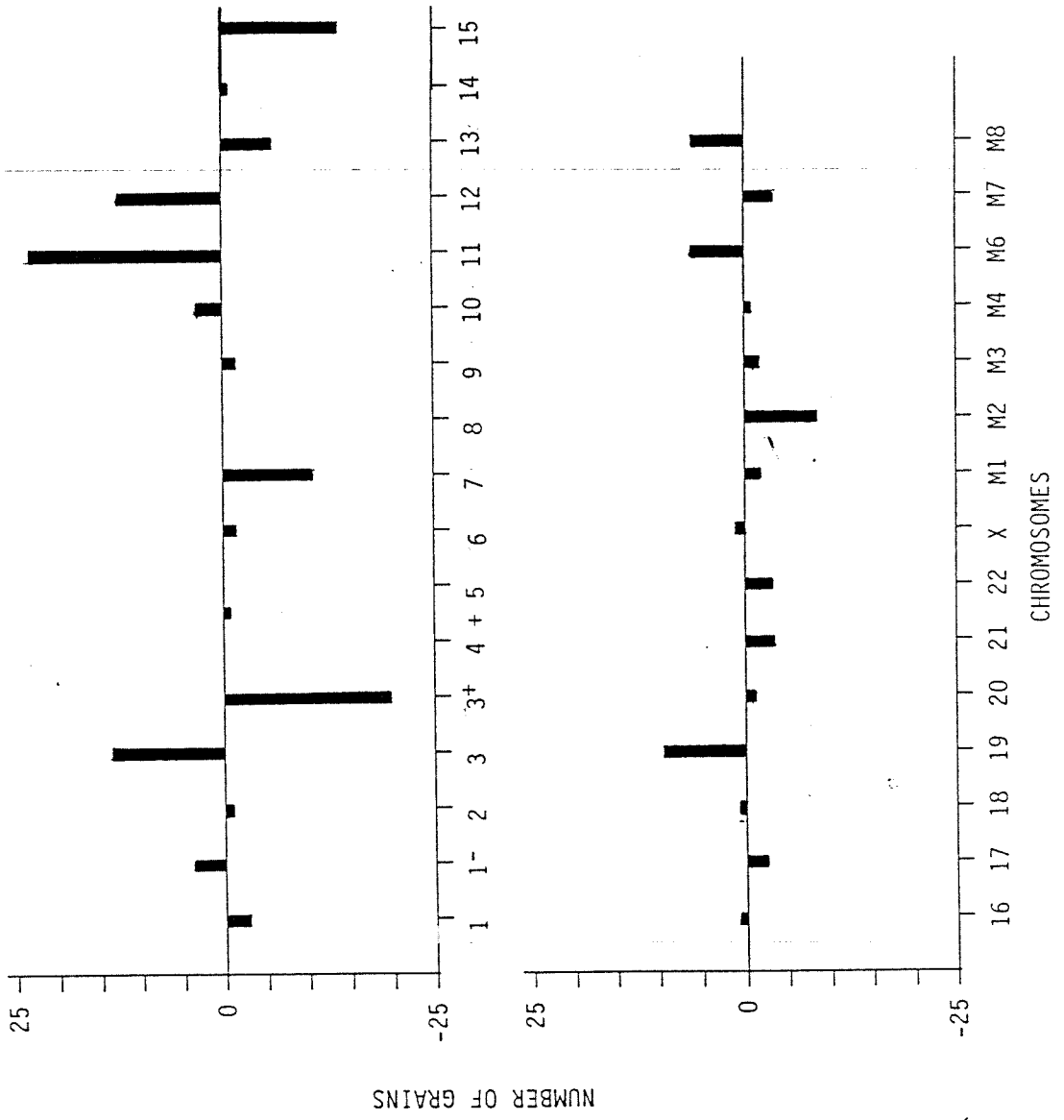


Figure 3-7a

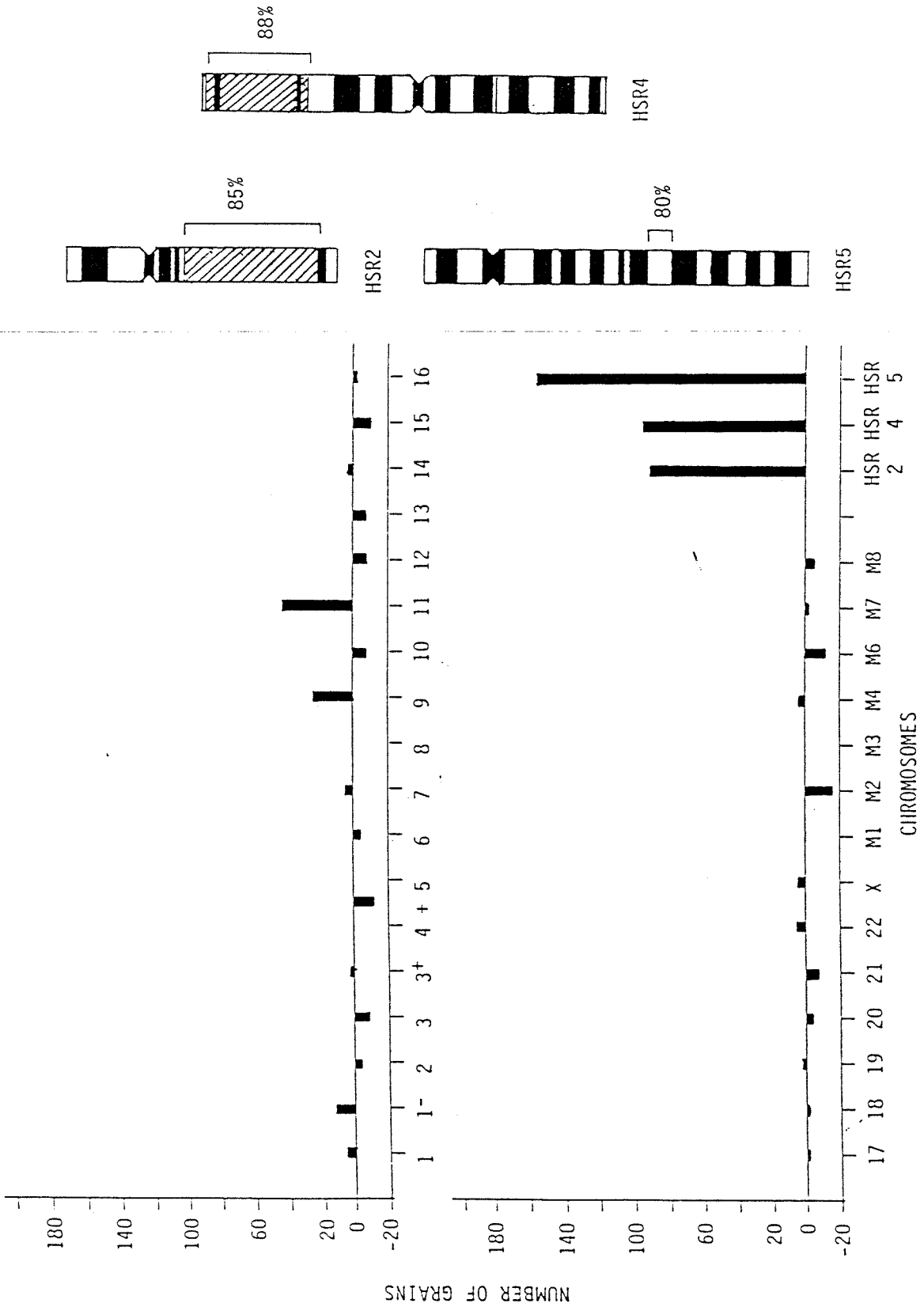


Figure 3-7b

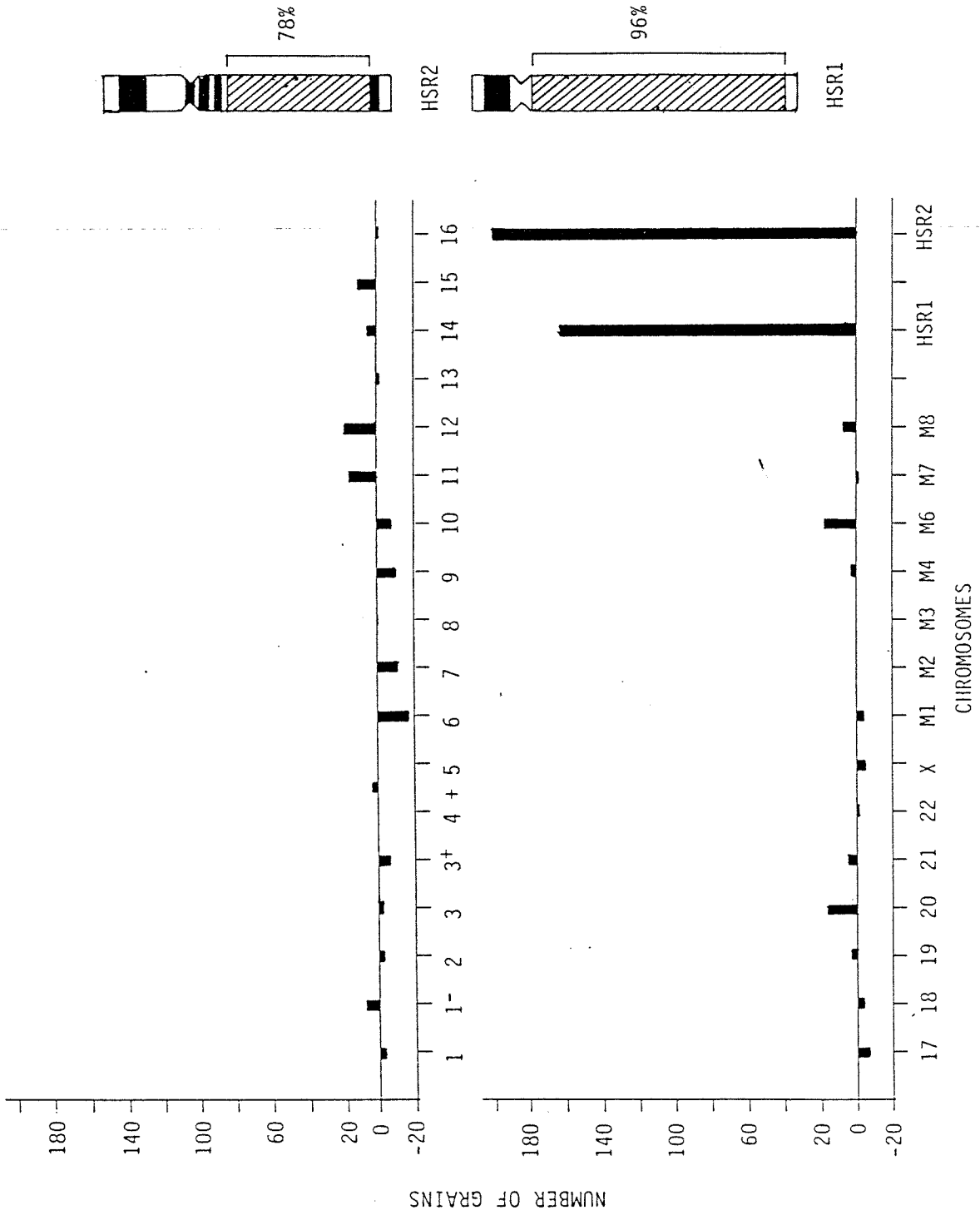


Figure 3-7c

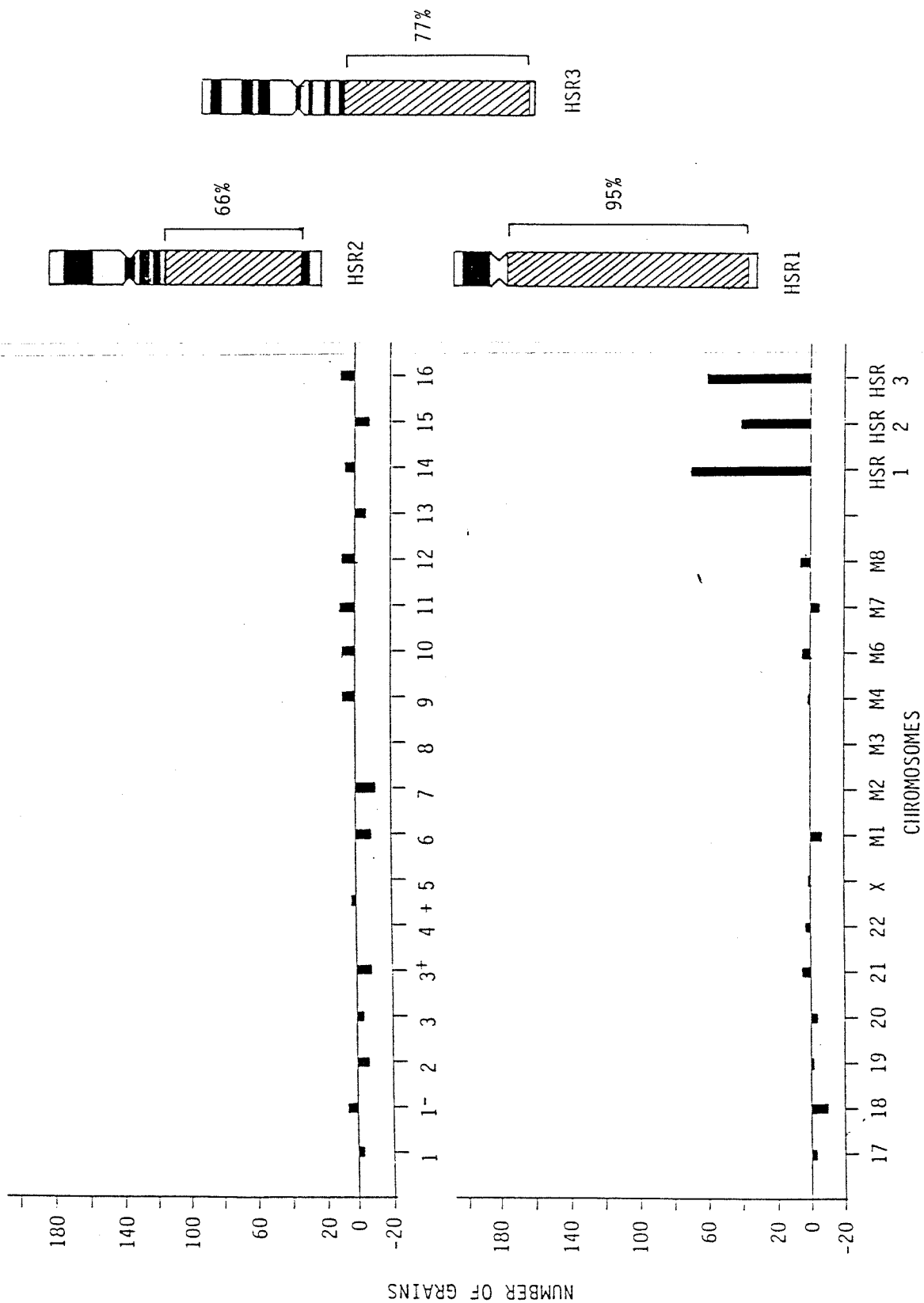


Figure 3-7d

CHAPTER FOUR

Dihydrofolate Reductase Gene Amplification in the Human Cell Sublines VA₂-6A3 A-E

DIHYDROFOLATE REDUCTASE GENE AMPLIFICATION IN THE HUMAN CELL SUBLINES VA₂-6A3 A-E

INTRODUCTION

Analysis of the evolution of the VA₂-6A3 MTX-resistant cell line revealed a complex relationship between the magnitude and stability of selective pressure, DHFR gene copy number, cell ploidy and the relative proportions of amplified genes in HSRs and those in minute chromosomes. Further, it was noticed (A. Chomyn, personal observation) that one of the HSR chromosomes, 6A3-HSR2, bore a striking resemblance to one found in another VA₂-B derived MTX-resistant variant, VA₂-7D2 (13). To better determine if the dynamics of gene amplification observed in VA₂-6A3 reflected a general rule for DHFR gene amplification in the VA₂-B cell line, and to establish if the evolution of 6A3-HSR2 was a predisposed, perhaps obligatory, event during that process, subpopulations from an early stage of VA₂-6A3 evolution (5×10^{-8} M D,L MTX) were subjected to stepwise selection in MTX and the chromosomal changes examined.

MATERIALS AND METHODS

Materials. MTX was obtained as (+)-Amethopterin (MTX) from Sigma Chemical Co. Concentrations will be listed as the corresponding concentration of (+ /-)-Amethopterin, so that results from previous work with VA₂-6A3 can be directly compared. Adverse metabolic effects of (-)-Amethopterin, which would preclude the comparison of the two forms of the drug (+/-):(+) on a 2:1 basis, have not been reported.

Cell lines and methods of growth. Five subpopulations (6A3 A-E) from frozen samples of VA₂-6A3, which had previously been grown in 5×10^{-8} M D,L MTX, were stepwise selected with increasing MTX concentration over 20–23 months. Resistance to 1.8×10^{-4} M D,L MTX ($= 9 \times 10^{-5}$ M (+) MTX) was obtained.

Chromosomal analysis. Chromosomal analysis was as described (Chapter Three). NOTE: In several sublines, variable numbers of very small minute chromosomes were observed at various stages of development. As it is difficult to distinguish very small minutes at low copy number from cellular debris, and to distinguish a single minute from a small paired double minute, recorded counts reflect conservative estimates.

Gene copy number estimations. DHFR gene-specific bands from Southern blots of EcoRI digested genomic DNA (6A3 A-E) probed with ³²P-labeled human DHFR cDNA were excised and counted in a scintillation counter, and the data were compared to Hela Bu25 DNA standards (see Methods, Chapter Three).

RESULTS

Chromosome constitutions. Metaphase spreads were Giemsa-banded and analyzed for chromosome number, minute chromosome number and for marker chromosomes (including HSRs) at the stages indicated in Tables 4-1a-e and Figure 4-1. Notable is the fact that four out of five subpopulations eventually became predominantly pseudotetraploid (2S), as was observed in 6A3. Further, subline 6A3-A developed a clear HSR on 11p similar to the 6A3HSR2

chromosome (marker chromosomes MA-HSR2, Figures 4-1 and 6-1). Subline 6A3-E had a chromosome 11 with a terminal addition to the short arm (an initial HSR?) (marker chromosome ME-4, Figures 4-1 and 6-1). Only one subline, 6A3-A, developed numerous HSR chromosomes as 6A3 did in the course of its evolution. These chromosomes appeared at approximately the same MTX concentration as did several in 6A3 (at 1.8×10^{-5} M D,L MTX) and, as also observed in 6A3, were subsequently eliminated from the population (including MA-HSR2, unlike its 6A3 counterpart). While in situ hybridization was not performed on these HSRs to confirm the presence of DHFR genes, the low level of minutes at the time points containing them makes this presence highly likely.

While four of five sublines exhibited more minutes at higher MTX concentrations, subline 6A3-B curiously exhibited a high number of minutes throughout the selection process. It was also observed that the minutes in 6A3-C and 6A3-D tended to be distinctly smaller in size than those observed in 6A3 (see Methods). Variability in minute size was observed in all sublines.

Analysis of marker chromosomes throughout the course of selection indicates that subclones within populations constantly appeared and were eliminated (data not shown). The occasional formation of chromosomal abnormalities, including chromosome fragments, endoreduplication, and rearrangements, were also observed in all lines, particularly at lower MTX concentrations (personal observation, see Chapter Six).

Estimation of gene copy number and comparison with chromosomal changes. The DHFR gene copy number for the final time points were estimated and summarized in Tables 4-1a-e. While there is general agreement between DHFR gene copy number and minute chromosome number,

it was observed that subline 6A3-B had generally high numbers of minutes regardless of the MTX concentration, and therefore it is possible that a fraction of the minutes in subline 6A3-B do not contain DHFR genes. Further, sublines 6A3-B and 6A3-E have been shown to contain a fraction of their DHFR genes on submicroscopic, extrachromosomal elements during their final stages (see Chapter Seven, sublines 6A3-A, -C and -D were not surveyed for submicroscopic elements). It is possible, therefore, that all sublines contained a fraction of their DHFR genes in this form at some or all stages of selection. The final DHFR gene copy number recorded for all sublines is roughly comparable to that observed in 6A3 (Month 8) before the development of HSR1 and HSR3 and the shift to a 2S chromosomal complement in 6A3. This suggests that resistance in all lines is due to amplification of DHFR genes whose products have equal enzymatic activity.

DISCUSSION

The observations on five subpopulations amplified from an early stage of the VA₂-B MTX-resistant clone, 6A3, indicate that the formation of an HSR does not appear to be an obligatory process in the development of high levels of MTX resistance in the VA₂-B cell line. While one of the five sublines, 6A3-A, developed numerous HSRs presumably containing DHFR genes as was observed in 6A3, other subpopulations, notably 6A3-C and 6A3-D, developed no obvious HSRs and limited numbers of marker chromosomes (which may have contained DHFR genes in "abnormally staining regions" (ABR) detectable only by in situ hybridization). While the presence of DHFR genes in ABRs in these sublines cannot be ruled out, the presence of minute chromosomes at all stages of development is adequate to explain the observed increase in drug resistance. It remains possible that HSRs could have developed

in all sublines but were eliminated before they were observed.

It is also to be concluded that there is a predisposition to the formation of an HSR on 11p in the VA₂-B cell line (see Chapter Six for further discussion) .

The variability in the chromosomal changes observed during the development of resistance in the five 6A3 subpopulations may reflect a stochastic process intrinsic to the amplification phenomenon or it may be a reflection of preexisting variability in the 6A3 population at the stage from which the sublines were amplified. The latter possibility is addressed in Chapter Five.

Lastly, four of the five populations exhibited a shift to a 2S chromosomal complement as was observed in 6A3. The exception, 6A3-D, contained a minority population with a 2S complement that increased in proportion at the last stage. As MTX uptake is mediated by a specific membrane transport system (68), this may reflect the growth advantage of cells containing lower (surface area / cell volume) ratios or (surface areas / gene complement) ratios, reflecting a more favorable ratio of (MTX uptake / DHFR synthetic capacity).

<u>[MTX]/Month</u>	<u>Chromosome Number (%)</u>	<u>Minute average Number (Range)</u>	<u>Marker Chromosome (%)</u>
7×10^{-7} M/5 (N=66)*	1S= 63 ± 3 (77) 2S= 125 ± 3 (18) 3S= 159, 193 (3) 4S= 253 (1)	44 ± 45 (2-281) 57 ± 40 (2-147) 137, 101 224	
6×10^{-6} M/7 (N=76)	1S= 62 ± 2 (92) 2S= 124 ± 4 (8)	27 ± 50 (0-289) 55 ± 51 (2-139)	MA1(41), MA2(2) MA6(43), MA14(70) MAHSR2(15)
1.2×10^{-5} M/9 (N=52)	1S= 62 ± 2 (71) 1.5S= 95 ± 12 (10) 2S= 122 ± 6 (13) 2.5S= 159 (2) 3S= 179 (2) 4S= 242 (2)	0.5 ± 2 (0-7) 0 0.5 ± 1 (0-2) 0 0 0	MA2(6), MA3(80) MA4(6), MA5(4) MA6(2), MA14(80) MAde11(4), MAHSR2(23)
1.8×10^{-4} M/18 (N=57)	1S= 61, 64 (4) 2S= 111 ± 5 (95) 4S= 210 (2)	0, 124 215 ± 144 (62-675) 200	MA14(84)
1.8×10^{-4} M/20 (N=32)	1S= 0 2S= 110 ± 6 (94) 3S= 171 (3) 4S= 217 (3)	310 ± 135 (104-707) 490 110	MA14 (84)

Final DHFR gene copy = 350 copies/cell

*number of metaphases analyzed

Table 4-1a. Chromosome changes in cell lines
VA₂-6A3 A-E during MTX selection.
VA₂-6A3-A

<u>[MTX]/Month</u>	<u>Chromosome Number (%)</u>	<u>Minute average Number (Range)</u>	<u>Marker Chromosome (%)</u>
1.4×10^{-6} M/5 (N=66)*	1S= 61 ± 3 (85) 2S= 116 ± 8 (14) 3S= 186 (2)	115 ± 108 (25-702) 175 ± 85 (88-339) 93	
6×10^{-6} M/7 (N=57)	1S= 59 ± 3 (79) 1.5S= 87,90 (4) 2S= $122 \pm$ (18)	237 ± 154 (29-521) MB1(9) 101,262 319 ± 302 (130-1000)	
1.8×10^{-4} M/15 (N=68)	1S= 60 ± 3 (93) 2S= 117 ± 4 (7)	192 ± 81 (5-439) MB1(4),MB2(69) 212 ± 137 (87-439)	
1.8×10^{-4} M/23 (N=62)	1S= 66 (2) 2S= 110 ± 4 (95) 4S= 203,214 (3)	164 $207 \pm$ (50-566) 264,247	MB2(63),MB3(26)

Final DFHR gene copy = 250 copies/cell

*number of metaphases analyzed

Table 4-1b. VA₂-6A3-B

<u>[MTX]/Month</u>	<u>Chromosome Number (%)</u>	<u>Minute average Number (range)</u>	<u>Marker Chromosome (%)</u>
1.4×10^{-6} M/5 (N=36)*	1S= 61 ± 2 (94) 1.5S= 94 (3) 2S= 116 (3)	92 ± 54 (28-330) 56 149	
1.2×10^{-5} M/11 (N=64)	1S= 60 ± 2 (98) 2S= 114 (2)	93 ± 60 (10-315) 26	
1.8×10^{-4} M/21 (N=32)	1S= 0 2S= 109 ± 7 (100)	191 ± 73 (92-353)	

Final DHFR gene copy = 300 copies/cell

*number of metaphases analyzed

Table 4-1c. VA₂-6A3-C

<u>[MTX]/Month</u>	<u>Chromosome Number (%)</u>	<u>Minute average Number (Range)</u>	<u>Marker Chromosome (%)</u>
7×10^{-7} M/5 (N=68)*	1S= 61 ± 2 (59) 1.5S= 90 (1) 2S= 120 ± 7 (32) 3S= 163 (1) 3.5S= 206,219 (3) 4S= 240 5S= 291	71 ± 40 (18-200) 167 141 ± 90 (46-330) 221 368,200 0 159	
1.2×10^{-5} M/12 (N=53)	1S= 60 ± 4 (98) 2S= 117 (2)	59 ± 44 (0-162) 41	
1.8×10^{-4} M/18 (N=55)	1S= 60 ± 2 (96) 2S= 116,120 (4)	284 ± 189 (21-1000) 737,600	MD1(6),MD2(2)
1.8×10^{-4} M/21 (N=36)	1S= 61 ± 4 (72) 2S= 117 ± 4 (28)	252 ± 98 (153-537) 471 ± 298 (180-1000)	MD3(61)

Final DHFR gene copy = 200 copies/cell

*number of metaphases analyzed

Table 4-1d. VA₂-6A3-D

<u>[MTX]/Month</u>	<u>Chromosome Number (%)</u>	<u>Minute average Number (Range)</u>	<u>Marker Chromosome (%)</u>
1.4×10^{-6} M/5 (N=64)*	1S= 60 ± 2 (98) 2S= 116 (1)	77 ± 62 (11-302) 88	
1.2×10^{-5} M/12 (N=51)	1S= $60 \pm$ (80) 2S= 115 ± 5 (20)	97 ± 63 (9-260) 163 ± 63 (78-275)	ME1(54),ME2(2)
1.8×10^{-4} M/21 (N=31)	1S= 63 (3) 1.5S= 84,90 (6) 2S= 105 ± 5 (87) 3S= 189 (3)	89 93,134 $147 \pm$ (75-290) 154	ME2(52),ME3(42)
1.8×10^{-4} M/23 (N=68)	1S= 75 ± 3 (4) 2S= 104 ± 7 (93) 2.5S= 158 (1) 4S= 216	239,225,346 206 ± 90 (76-535) 117 543	ME2(84),ME3(51) ME4(3)

Final DHFR gene copy = 300 copies/cell

*number of metaphases analyzed

Table 4-1e. VA₂-6A3-E

Figure Legends

Figure 4.1. Marker chromosomes observed throughout MTX selection of cell lines VA₂-6A3-A-E. Bar represents equivalent length of chromosome 1. In VA₂-6A3-A, chromosome MAdel1 bears an HSR on 1q-; MAHSR2 resembles the 11p+ HSR observed in VA₂-6A3; MA14 resembles a 14q+.

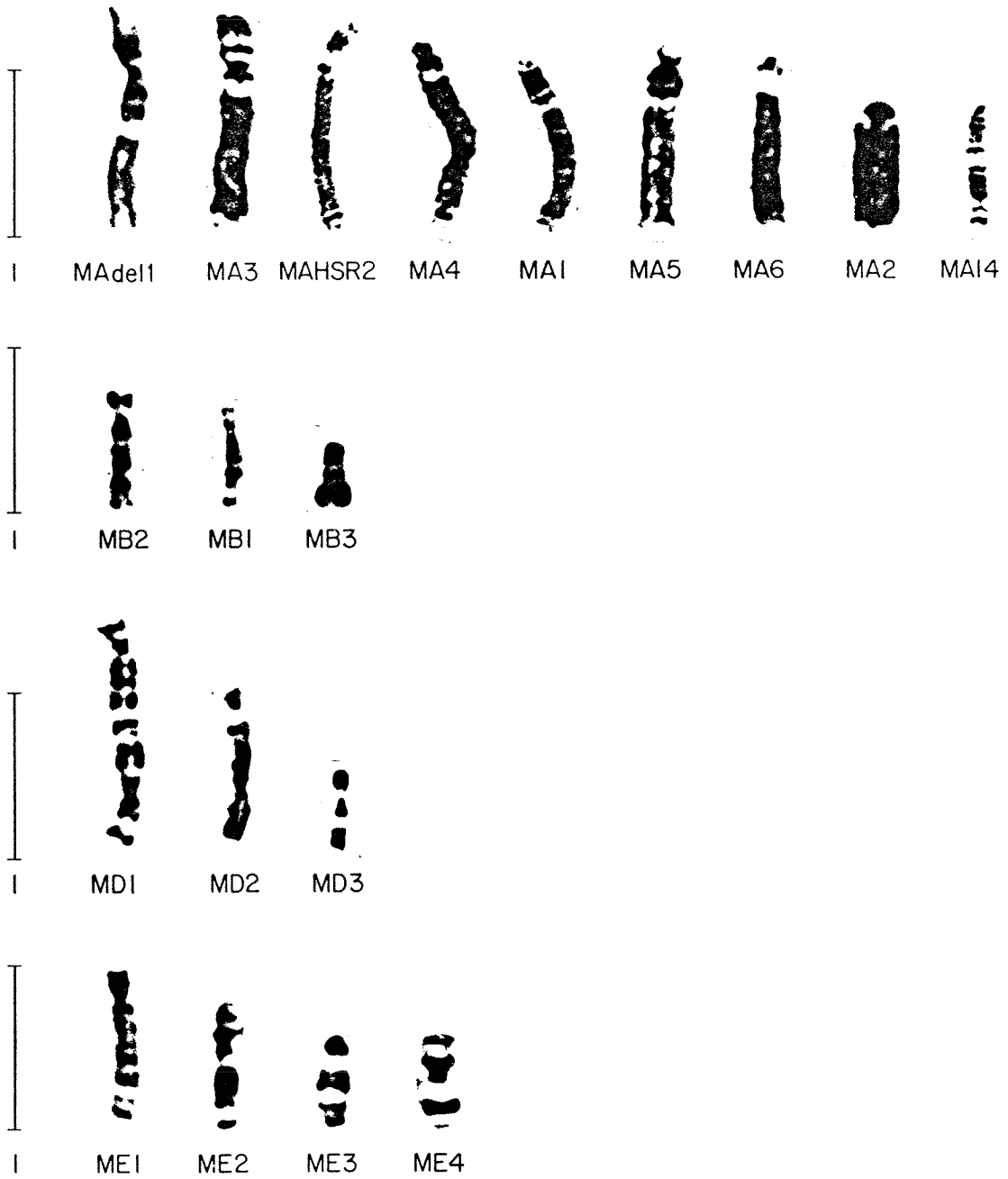


Figure 4-1

CHAPTER FIVE

Dihydrofolate Reductase Gene Amplification in the Human Cell Sublines VA₂-6A3-A'

DIHYDROFOLATE REDUCTASE GENE AMPLIFICATION IN THE HUMAN CELL LINE VA₂-6A3-A'

INTRODUCTION

Analysis of the VA₂-B MTX-resistant cell lines 6A3 and 6A3-A-E revealed heterogeneity in the chromosomal changes that occurred during stepwise selection and continued growth. In order to assess whether this variation was due to preexisting genetic variation within the 6A3 population or reflected a variability in the processes leading to DHFR gene amplification and MTX resistance, a clone of 6A3 (labeled 6A3-A') from the 5×10^{-8} M D,L MTX stage was isolated, and five subpopulations of 6A3-A' subjected to stepwise selection in MTX.

MATERIALS AND METHODS

Materials. MTX was obtained from Sigma Chemical Co. as (+)-Amethopterin. MTX concentrations are expressed as the corresponding concentration of D,L MTX.

Cell lines and methods of growth. A subclone from the 6A3 population (labeled 6A3-A') was isolated at the 5×10^{-8} M D,L MTX stage and five subpopulations (6A3-A'1-5) were stepwise selected to resistance to 1.8×10^{-4} D,L MTX over 12–15 months.

Chromosomal analysis. Analysis was as described previously. All cell subpopulations were examined at one early stage (1.8×10^{-6} M MTX) and

three (6A3-A'2, -A'3 and -A'5) were examined at a late stage (1.8×10^{-4} M MTX).

Estimation of DHFR gene copy number. Genomic DNAs (6A3-A'2, -A'3 and -A'5) were isolated and digested with EcoRI restriction endonuclease, and Southern blots were probed with the human DHFR cDNA containing plasmid, pHD84. Densitometry tracings of the autoradiograms were compared with Hela Bu25 DNA standards (see previous).

RESULTS

Chromosome constitutions and DHFR gene copy numbers. The chromosomal constitutions and DHFR gene copy numbers for the clone 6A-3A' and subpopulations 6A3-A'1-5 at the stages indicated are shown in Table 5-1a-f. A small percentage of the 6A3-A' parental clone exhibited a 2S or 3S complement soon after isolation. 6A3-A'1 exhibited a predominantly 2S complement at a comparatively early stage, while all three subpopulations analyzed at the 1.8×10^{-4} M MTX stage possessed 2S complements. No subline exhibited obvious HSRs at the stages analyzed. One, 6A3-A'1, exhibited a conspicuous marker chromosome (not shown). Overall, DHFR gene copy numbers were lower in the three subpopulations at the 1.8×10^{-4} M MTX stage than in 6A3 or 6A3-A-E analyzed at similar stages.

DISCUSSION

This study attempted to assess the role of a possible preexisting genetic heterogeneity in the populations previously examined (6A3, 6A3-A-E) as a

cause for the variability in chromosomal changes observed during their adaptive selection in MTX. Of principal concern was whether clonal subpopulations would evolve HSRs similar to those observed in 6A3 and 6A3-A-E (in particular, an HSR on 11p), and whether the same HSRs would develop in all subpopulations. The apparent lack of HSRs at the stages examined and the paucity of marker chromosomes may indicate a greater inherent chromosomal stability in the 6A3-A' clone as compared to the population from which it was derived, or may be a reflection of the greater genetic homogeneity of the 6A3-A' subpopulations resulting from cloning. (One would expect a heterogeneity in the 6A3 population at the 5×10^{-8} MMTX stage from which all cell lines in these studies were derived, as the most sensitive fragile sites in the chromatin induced by inhibitors of folate metabolism were presumably stressed and chromosomal fragmentation and rearrangement occurred. Clones derived from the 6A3 population at this stage might be expected to exhibit greater chromosomal stability, as only less sensitive fragile sites would remain). If the chromosomal changes observed in 6A3 and 6A3-A-E were due solely to (possibly) stochastic processes involved in DHFR gene amplification, then it would be expected that these processes would also be observed in 6A3-A'1-5. That no obvious HSRs developed, especially at 11p, in 6A3-A'1-5 (although lacking in situ hybridization data and examination at as many stages) may indicate that the predisposition to form an HSR at 11p is a reflection of an early expansion of subpopulation(s) with 11p damaged in such a way that favors the development of an HSR. The latter event, therefore, is not involved in an obligatory way in the gene amplification process itself.

The development of minute chromosomes and of a change to a 2S chromosomal complement would seem to be fundamental manifestations of the amplification and selection processes in VA₂-B cells exposed to increasing concentrations of MTX, as they remain common features of the 6A3-A' subpopulations as well as of the 6A3 and 6A3-A-E populations.

<u>[MTX]/Month</u>	<u>Chromosome Number (%)</u>	<u>Minute average Number (Range)</u>	<u>Marker Chromosome (%)</u>
5×10^{-8} M/0 (N=72)*	1S= 64 ± 3 (93) 2S= 123 ± 3 (6) 3S= 167 (1)	12 ± 12 (0-31) 7 ± 8 (0-18) 4	

Table 5-1a. Chromosomal changes in cell sublines
VA₂-6A3-A' 1-5 during MTX selection.
VA₂-6A3-A' Parental

<u>[MTX]/Month</u>	<u>Chromosome Number (%)</u>	<u>Minute average Number (Range)</u>	<u>Marker Chromosome (%)</u>
1.8×10^{-6} M /5 (N=63)*	1S= 63,78 (3) 2S= 123 ± 9 (83) 3S= 180 (1) 4S= 241 ± 28 (11) 5S= 317	6,2 13 ± 15 (0-73) 22 33 ± 29 (6-75) 14	MA'1-1(50) MA'1-1(56) MA'1-1(43)

*number of metaphases analyzed

Table 5-1b. VA₂-6A3-A'1

<u>[MTX]/Month</u>	<u>Chromosome Number (%)</u>	<u>Minute average Number (Range)</u>	<u>Marker Chromosome (%)</u>
1.8×10^{-6} M/4 (N=58)*	1S= 61 ± 4 (83) 2S= 115 ± 9 (16) 3S= 182	11 ± 12 (0-36) 18 ± 11 (5-33) 18	
1.8×10^{-4} M/11 (N=34)	1S= 62,81 (6) 2S= 116 ± 4 (88) 3S= 184 (3) 4S= 231 (3)	162,63 227 ± 191 (22-711) 232 134	

Final DHFR gene copy = 220 copies/cell

Table 5-1c. VA₂-6A3-A'2

<u>[MTX]/Month</u>	<u>Chromosome Number (%)</u>	<u>Minute average Number (Range)</u>	<u>Marker Chromosome (%)</u>
1.8×10^{-6} M/6 (N=67)*	1S= 63 ± 3 (79) 2S= 124 ± 9 (19) 3S= 165	8 ± 6 (0-32) 17 ± 13 (0-42) 15	
1.8×10^{-4} M/15 (N=36)	1S= 79 (3) 2S= 123 ± 2 (97)	47 128 ± 64 (60-363)	

Final DHFR gene copy = 160 copies/cell

*number of metaphases analyzed

Table 5-1d. VA₂-6A3-A'3

<u>[MTX]/Month</u>	<u>Chromosome Number (%)</u>	<u>Minute average Number (Range)</u>	<u>Marker Chromosome (%)</u>
1.8×10^{-6} M/4 (N=64)*	1S= 64 ± 6 (91) 2S= 120 ± 12 (10)	11 ± 10 (0-46) 17 ± 12 (4-32)	

Table 5-1e. VA₂-6A3-A'4

<u>[MTX]/Month</u>	<u>Chromosome Number (%)</u>	<u>Minute average Number (Range)</u>	<u>Marker Chromosome (%)</u>
1.8×10^{-6} M/5 (N=64)*	1S= 64 ± 9 (83) 2S= 117 ± 10 (17)	12 ± 12 (0-23) 18 ± 23 (3-80)	
1.8×10^{-4} M/15 (N=35)	1S= 0 2S= 101 ± 4 (100)	138 ± 57 (43-281)	

Final DHFR gene copy = 100 copies/cell

*number of metaphases analyzed

Table 5-1f. VA₂-6A3-A'5

CHAPTER SIX

**Karyotypic Abnormalities Observed During Dihydrofolate
Reductase Gene Amplification in the Human Cell Lines
VA₂-6A3, 6A3-A-E and 6A3-A'1-5**

**KARYOTYPIC ABNORMALITIES OBSERVED DURING GENE
AMPLIFICATION IN THE HUMAN CELL LINES
VA₂-6A3, 6A3-A-E AND 6A3-A'1-5**

INTRODUCTION

The purpose of studying the karyotypic changes that occurred in VA₂-6A3 and its related cell lines during DHFR gene amplification was to determine if these changes could lend direct or indirect insight into the molecular mechanisms of gene amplification in human cells. Overall, a number of changes were observed that were common to all cell lines. It was observed that chromosomal fragmentation occurred in all lines at some level, predominantly at early stages of selection (data not shown). These fragments could occasionally be identified as deriving from a specific chromosome, but usually could not. Fusion events between chromosomes were observed at all stages of selection and cell spreads often exhibited multiple fusion events. There was no stage specificity observed for fusion events. Endoreduplication (see below) was observed at a low level (less than 1% to several percent depending on cell line and stage) in all lines. Prematurely condensed chromatin (PCC) occurred at low, but higher, levels than endoreduplication in all cell lines. Lastly, as previously noted, all cell lines exhibited a tendency to shift to a 2S chromosomal complement. The predisposition to the formation of an HSR on 11p has also been mentioned. These findings and their mechanistic implication are discussed below.

The Formation of an HSR on 11p.

The most singular observation on the 6A3 cell lines was the tendency to form an HSR at about 11p14.2. Initially, it was noted (A. Chomyn, personal observation) that a similarity existed between two HSR chromosomes derived from 6A3 and another VA₂-derived MTX-resistant line, 7D2. In the course of this study, similar HSRs were observed on 11p in 6A3-A, 6A3-E and in another cell line isolated in this laboratory, 6A2 (Figure 6-1). While both 6A3 and 6A2 derive from a common parent, 6A (see Methods, 13), the 7D2 line does not and therefore the predisposition to form an HSR on 11p would seem to be an inherent characteristic of the VA₂-B cell line that is induced by MTX selection. The significance of this is not clear.

Consistent HSR formation at the native locus of a gene has been reported in rodent cell lines (see Introduction), but the normal human DHFR gene resides at 5q11-q23, not at 11p14.2. The formation of HSRs at genomic locations other than at the native gene site is common (3,6) but the predisposition to form an HSR at another specific chromosome site has not been previously reported.

The VA₂-B line is known to contain a moderate amplification of DHFR genes (see Chapter Three). However, as these copies were lost at the initial selection stage prior to the formation of an HSR on 11p in the 6A3 line (Figure 3-2) and as in situ hybridization fails to localize these copies to 11p in VA₂-B (Figure 3-7a), it would seem unlikely that the formation of HSRs on 11p reflects the amplification of DHFR genes previously transposed to that site in VA₂-B.

It is noted that a folate deficiency sensitive site exists at 11p14.2 (75). It is possible that the existence of this sensitivity predisposes to HSR formation.

However, there exist numerous folate deficiency sensitive sites in the genome and it is not clear why HSR formation would not be preferentially observed at them as well.

Formation of Ring Chromosomes and Chromosome Fusions.

The formation of ring chromosomes composed of amplified-gene containing chromatin has been reported in murine cell lines (77). The occurrence of this phenomenon in the human cell lines examined was very rare. A singular case was observed in 6A3-A (Figure 6-2 panel A). The ring chromosome appears to derive from end-to-end fusion of several 6A3-A MA3 chromosomes and is longer in length than the sum of two 6A3-A MA3 chromosomes. The complex nature of this fusion event would seem to make it questionable that it could have survived a previous mitotic event intact (allowing unequal sister chromatid exchange in subsequent generations to increase its size) and therefore suggests that HSRs can be amplified to a considerable extent within a single cell generation. This is in keeping with the previously noted observation that HSRs appeared "catastrophically" in the population (Chapter Three), neither expanding or shortening in length over time. These observations imply that unequal sister chromatid exchange is not a driving mechanism in at least the later stages of HSR formation. If it were, HSRs would have been observed to lengthen during the course of their evolution. It would also be difficult to explain the fusion event in Figure 6-2A by this mechanism. Such a chromosome may possibly be explained by the initiation of intrachromosomal, double rolling circle replication following the formation of an end-to-end fusion

chromosome. It would, however, be extremely difficult to document such an event on the molecular level. Similar end-to-end fusions of other HSR containing chromosomes (Figure 6-2 panel B), some exhibiting an expanded HSR region that was larger than the sum of the participating HSR chromosomes, were occasionally observed in other cell lines, principally 6A3 at later stages (data not shown). Ring chromosomes formation was absent in these cases.

Endoreduplication and Endomitosis.

While it has been recently reported that molecular evidence for reinitiation of DNA synthesis at a specific locus within a single cell cycle is lacking (62), it has long been known that mammalian cells (in vitro cultured lines), in tumors and in embryonic development possess the ability, under certain circumstances, to amplify their entire genome many fold within a single, extended cell cycle through the processes of endoreduplication (78-83) and endomitosis (84).

Endoreduplication is a process wherein multiple rounds of DNA synthesis can occur between normal S phase and mitosis. In this process, cells exiting a normal S phase (S_1) enter an interperiod termed G_7 and then proceed to further consecutive synthetic phases (S_2 and S_3). In humans, an abnormally high incidence of endoreduplication is associated with Bloom's syndrome (85). Further, various chemical mutagens have been shown to induce either full or partial endoreduplication of the genomic complement. Chromosomal aberrations including sister chromatid exchange and arch structures can occur as a result of this process (86,87). Therefore, mammalian cells possess the capacity to amplify all or large parts of their genome in a single cell cycle (between two mitotic

events).

Endoreduplication was repeatedly observed in all the MTX-resistant cell lines in this study (Figure 6-3), in some lines at levels several times that of the VA₂-B parental line (personal observation). While the relevance of endoreduplication to the amplification of specific genes is not clear, it does provide the cell a way to amplify required genes several fold. Unequal sister chromatid exchange after several rounds of DNA synthesis might possibly result in an initial amplification sufficient to overcome MTX inhibition and would be manifested as a contiguous sequence of the rearranged genes that could resemble an HSR or ABR chromosome. However, resolution of the chromosome complement by this mechanism would be complex.

Endomitosis is a process where the cell undergoes successive rounds of DNA synthesis without a breakdown of the nuclear envelope and the development of a spindle apparatus. In contrast to endoreduplication, in endomitosis chromosomes condense to form mitotic figures within the nucleus before decondensing to undergo further rounds of synthesis. Very high genomic complements can be obtained by this process (78) and endomitosis has been reported in the normal human trophoblast (84).

Which of the above two processes may be responsible for the 2S complement shift observed in all cell lines is unknown.

Another mechanism for the generation of a 2S complement in cancer cells, termed restitution (C-mitotic duplication), has also been reported (84). This process involves nondisjunction of the entire genome at cell division.

Formation of Premature Condensed Chromatin (PCC) in 6A3 - Derived Lines.

Premature Condensed Chromatin (PCC) is a phenomenon occurring in multinucleate and micronucleate cells and in heterokaryons wherein a mitotic nucleus induces the breakdown of the nuclear envelope in a nonmitotic nuclei (88). The form of the PCC observed is related to the cell cycle of the induced nucleus. Induced nuclei previously in G₁ exhibit very diffuse chromatin, while those in S phase exhibit PCC in various stages of "pulverization." "Pulverized" DNA in early and late S phase exhibits a "beads-on-a-string" appearance with "beads" of condensed chromatin connected by "strings" of replicating DNA (89). PCC in mid-S phase exhibits the greatest degree of pulverization with the chromatin appearing totally granular (88). PCC in S phase has been shown to segregate randomly at subsequent cell divisions while that in G₁ and G₂ stages have been shown to be excluded from the spindle apparatus and to be lost through micronucleation (90).

The significance of PCC to gene amplification can be observed in Figure 6-4b. The premature condensation of S phase DNA, presumably induced by the observed mitotic figure, has resulted in pulverized DNA that bears extraordinary resemblance to DMs. It has been suggested (91) that PCC derived from HSRs could be the origin of the DMs observed after fusion of a tumor cell line bearing an HSR with a "normal" cell line. However, it should be apparent that PCC DNA could be the origin of DMs in a cell without a preexisting HSR. The retention of PCC fragments conferring selective growth advantage in either tumors or drug-selected cell lines would result in a cell with an extrachromosomal gene complement that could be increased by asymmetric segregation in subsequent

mitoses or could incorporate into a chromosome to form the origin of an HSR. The postmitotic stability of PCC is not known however. Lacking proper telomeric structures, the PCC may be totally degraded, microenucleated or diluted out.

The occurrence of PCC in all cell lines studied here indicates that it could be an initiating event in gene amplification. Comparison of the frequency with which genes can be selectively amplified in normal cells versus fusion-induced, PCC-containing cells would be extraordinarily informative in determining the role that this phenomenon may play in the amplification process both in vitro and in vivo. The observation that DMs are found predominantly in solid tumors may perhaps relate to a possible increase in the probability of cell fusion (inducing PCC formation) in solid tumors versus leukemias or ascite tumors.

Multiple Karyotypic Abnormalities as Evidence of Genomic Plasticity.

The mechanistic implications of chromosome fusion, endoreduplication and PCC as described above are unclear. However, the extraordinary metaphase spread shown in Figure 6-4a demonstrates that these processes may operate simultaneously in the amplification process. The spread displays an increased chromosome complement, DMs, numerous chromosome fusions and what appears to be a partial complement of DNA in a state of PCC interspersed with chromatin fragments and DMs. Whether or not the PCC DNA represents bulk chromatin or concatenated DMs containing DHFR genes is undetermined. However, it is obvious that the spread displays a remarkable plasticity of the genome. This plasticity is independent of unequal sister chromatid exchange and would not appear to represent *Drosophila*-type onionskin overreplication.

Whether this spread represents a cell in the process of de novo amplification or one that has been terminally perturbed cannot be determined. Its fate upon completing mitosis must remain speculative. If, however, this spread represents a cell in the process of increasing its fitness to selective pressure, it is clear that unequal sister chromatid exchange or onionskin overreplication need not be invoked to explain the process.

Figure Legends

Figure 6-1. 11p⁺ HSR chromosomes observed in several MTX-selected VA₂-B derived cell lines. A, chromosome 11 from VA₂-B; B, HSR-11p⁺ from VA₂-6A2 (13); C, HSR2 from VA₂-6A3; D, MAHSR2 from VA₂-6A3-A; E, chromosome 11; F, chromosome 11 from VA₂-6A3-E; G, 11p⁺ from VA₂-6A3-E. (See Figure 4-1 for explanation of chromosome symbols).

Figure 6-2. Ring chromosome and chromosome fusion events observed in several MTX-selected VA₂-B-derived cell lines. a, ring chromosome and fusion events involving MA3 in subline VA₂-6A3-A, Month 9. Thin arrows indicate MA3 chromosomes. Thick arrow shows possible ring chromosome. b, HSR fusion and fragmentation events in VA₂-6A3, Month 20. Thick arrow shows end-to-end HSR fusion of HSR2. Thin arrow shows HSR region without accompanying centromeric region, origin unknown, possibly secondary to chromosome bridge-fusion event involving HSR2.

Figure 6-3. Endoreduplication in cell line VA₂-6A3-D, Month 5. Overexposure of photograph would demonstrate DMs.

Figure 6-4. Metaphase spreads demonstrating abnormal chromatin and premature condensed chromatin (PCC). a, Metaphase spread from VA₂-6A3-E, Month 12. Thick arrow demonstrates extended chromatin connecting DMs and pulverized chromatin. Thin arrows show DMs and an extended region of abnormal chromatin. The spread also demonstrates several fusion events. b, Metaphase spread from VA₂-6A3-A'1, Month 5. Spread shows pulverized PCC

DNA presumably induced by the overlaying metaphase figure. Note resemblance of PCC DNA to DMs.

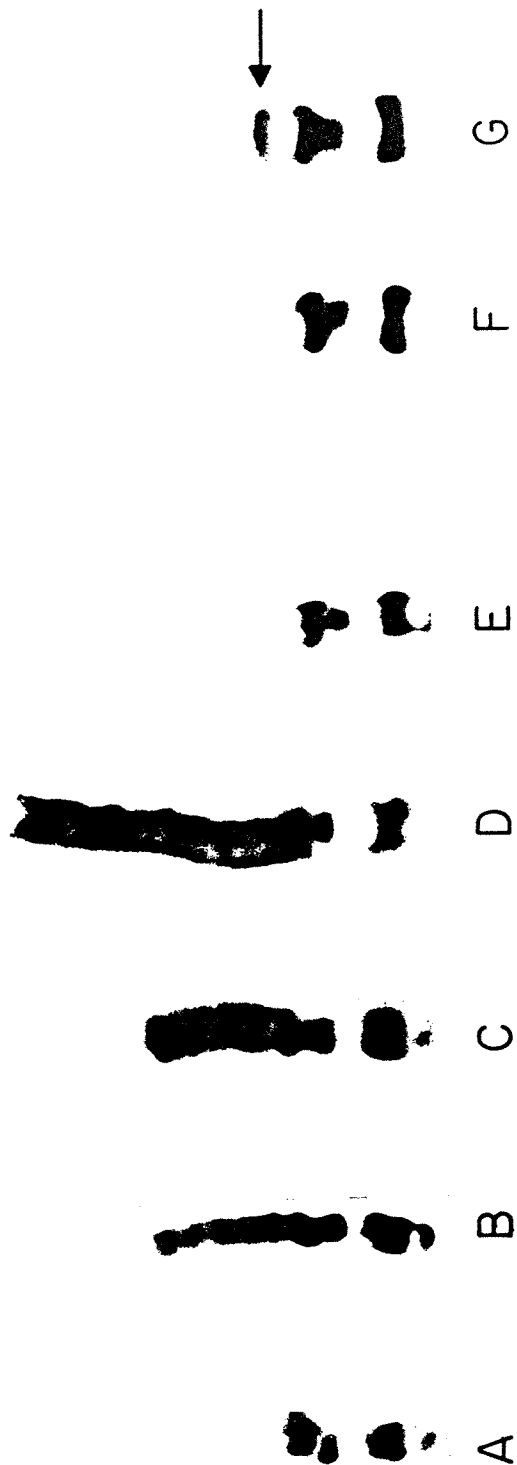


Figure 6-1



Figure 6-2

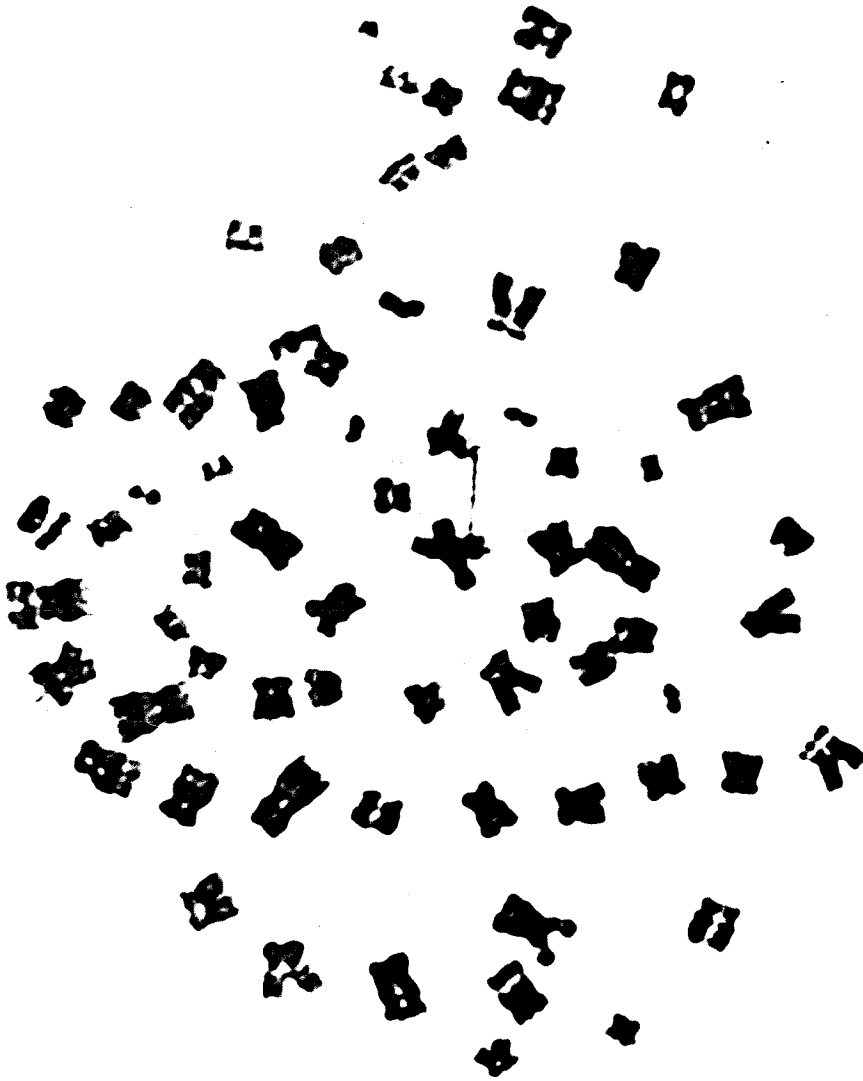


Figure 6-3



A



B

Figure 6-4

CHAPTER SEVEN

Novel Submicroscopic Extrachromosomal Elements Containing Amplified Genes in Human Cells

Novel submicroscopic extrachromosomal elements containing amplified genes in human cells

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In previous work¹, several methotrexate (MTX)-resistant variants were isolated from the human cell line HeLa BU25, which exhibited a high degree of dihydrofolate (DHFR) gene amplification (estimated to be 250- to 300-fold)^{1,2}. These variants did not contain any chromosome with a homogeneously staining region (HSR) and exhibited only a small average number of minute chromosomes per cell: these two types of karyotypic abnormalities generally accompany selective gene amplification³. We now report that structures containing amplified DHFR genes in one of these variants (HeLa BU25-10B3) can be isolated by pulsed-field gradient or field-inversion gel electrophoresis as homogeneous DNA molecules of ~650 kilobases (kb). Electron microscopy of metaphase spreads from these cells reveals chromatin fibres with a similar DNA content, which are probably related to the above elements. These represent a novel type of extrachromosomal structures in mammalian cells.

An experiment in which a metaphase chromosome preparation from 10B3 cells was fractionated in a sucrose gradient, and DNA from the individual fractions was tested with chromosome-specific probes showed that most of the DHFR-specific sequences are in structures sedimenting slower than chromosome 5, which contains the unamplified DHFR gene⁴, and also slower than chromosomes 13, 14, 15, 21 and 22, which contain the

nuclear ribosomal RNA genes⁵ (data not shown). This experiment indicated that the amplified DHFR genes in these cells are in extrachromosomal structures not visible in the light microscope due to their small size.

Electrophoretic techniques using orthogonal^{6,7} or inverted-field pulses⁸ permit the separation of large DNA in the range 50-2,000 kb, such as the intact chromosomal DNAs from yeast^{6,7,9} and protozoa¹⁰⁻¹², and offered the possibility of isolating the putative submicroscopic extrachromosomal elements. Samples of genomic DNA from HeLa S3 and HeLa BU25-10B3¹, and, for comparative purposes, from the MTX-resistant human cell variants VA₂B-6A₂-H¹, VA₂B-6A₃-B and VA₂B-6A₃-E, which contain abundant minute chromosomes (see legend of Fig. 1 and Table 1), were run in the pulsed-field gradient electrophoresis (PFGE) system (Fig. 1a,b). The ethidium bromide (EtBr) staining pattern (Fig. 1a) reveals, in the yeast DNA lanes, bands corresponding to individual chromosomes or unresolved chromosome doublets⁷⁻⁹. In the 10B3 DNA lane, a discrete band (thick arrow), absent in the other lanes, has migrated to a position between those of chromosomes V and VIII (~580 kb) and chromosome XI (~700 kb)⁹, and thus has a mobility corresponding to that of linear DNA of ~650 kb. The 10B3 DNA lane also contains fairly abundant DNA of heterogeneous size, of <300 kb, which possibly derives, in part at least, from degradation of larger molecules. The DNA samples from 6A₂, 6A₃-B and 6A₃-E cells show a band migrating approximately as yeast chromosome X (thin arrow), which is absent from the HeLa and 10B3 DNA samples, and which may be specific for the cell line VA₂-B and its derivatives.

The '650-kb' band of the 10B3 genomic DNA sample hybridized strongly with the human DHFR complementary DNA probe (pHD84; ref. 2), whereas the <300 kb heterogeneous DNA gave a much weaker signal with this probe (Fig. 1b). The 650-kb band most probably represents the extrachromosomal structures containing amplified DHFR genes revealed by the sucrose gradient fractionation experiment. Note that the minute chromosome DNA from this cell line and from the 6A₃-B, 6A₃-E and 6A₂ variants has not migrated from the origin.

Fig. 1 Pulse field gradient gel electrophoresis (PFGE) of genomic DNA isolated from different human cell lines, MTX-sensitive (HeLa S3) or MTX-resistant (HeLa BU25-10B3, VA₂-6A₂, VA₂-6A₃-B, VA₂-6A₃-E), or from *S. cerevisiae* (SS327), and detection of chromosomal structures containing amplified DHFR genes. **a** and **c**, EtBr-stained gels; **b**, DNA blot of the gel shown in **a** probed with the human DHFR cDNA clone pHD84². The gel shown in **a** and **b** was run with a switching interval of 90 s; that in **c**, with a switching interval of 60 s. The human cell variant VA₂-6A₂, which had been grown in 1.8×10^{-4} M DL-MTX for about one year, has been previously described¹; the cell lines VA₂-6A₃-B and VA₂-6A₃-E were derived by subjecting two samples of a precursor population of VA₂-6A₃ (ref. 1) at an early stage of the DHFR gene amplification process (resistant to 1×10^{-7} M MTX) to progressively increasing concentrations of the drug, up to 1.8×10^{-4} M, and subsequently maintained at this concentration for 11 and 7 months, respectively (unpublished data).

Methods. The cells were harvested by trypsinization, washed with phosphate-buffered saline (PBS) (without magnesium and calcium) at 37 °C, resuspended at 4×10^7 cells ml⁻¹, and gently mixed with an equal volume of a 1% solution of low-melting agarose (Seaplaque, FMC Bioproducts) in Mg²⁺, Ca²⁺-free PBS precooled to 37 °C. The agarose-cell mixture was allowed to solidify in the cold, and agarose-cell plugs were then treated with proteinase K (2 mg ml⁻¹) in the presence of 0.5% Sarkosyl, washed extensively, and finally treated with RNase A (100 µg ml⁻¹), under conditions to be described in detail elsewhere. After washing, the plugs were stored at room temperature in 10 mM Tris buffer, pH 8.0, 50 mM NaCl, 10 mM EDTA. Agarose plugs containing DNA from *S. cerevisiae* (SS327) were prepared as described by Schwartz and Cantor⁶. PFGE was carried out with a modification of the apparatus described by Carle and Olson⁷ (E. Lai, R. Barth & L. Hood, manuscript in preparation), using 1% agarose gels (SeaKem ME agarose, from FMC Bioproducts) in 0.5 × TBE buffer (TBE: 90 mM Tris borate, 90 mM boric acid, 2 mM EDTA, pH 8.0) and a 0.1-ml portion of the desired agarose-DNA plug (~20 µg DNA from the human cell lines). Electrophoresis was run at 10 V cm⁻¹ for 36 h (12°-14°C) with the switching time specified above, using recirculating 0.5 × TBE. After EtBr staining and UV visualization, the agarose gels were processed for DNA transfer by exposure to short-wave UV light for 60 s, soaking in 0.4 N NaOH for 15 min and blotting onto nylon membranes (Zeta-Probe, Biorad Laboratories) for 2 days in the presence of 0.4 M NaOH. After a brief exposure to 0.5 M Tris buffer, pH 8.0, 1.5 M NaCl, and baking in a vacuum oven, the filters were treated for hybridization with a ³²P-labelled probe according to standard procedures.



As shown in Fig. 1c, changing the PFGE pulse length from 90 s to 60 s considerably affected the migration of the yeast chromosomal DNAs. The migration of the 10B3 650-kb elements was similarly affected. As it is known that small closed-circular DNAs, such as the supercoiled 2 μ circles of yeast (6.3 kb) and supercoiled 45-kb cosmid DNAs, unlike linear DNAs, exhibit a mobility in PFGE which is independent of the pulse times (refs. 6, 12; E. Lai, unpublished data), the above observations strongly argued against the 650-kb elements being small supercoiled circles (see also below).

For an independent estimate of the size of the 650-kb elements, they were digested with the restriction enzyme *Sfi*I, and probed with nick-translated 650 kb DNA (Fig. 2). Both the EtBr staining pattern (Fig. 2a) and the autoradiogram (Fig. 2b) show three bands corresponding to components of ~370 kb, ~125 kb and ~75 kb in size, with no smaller fragment appearing in a shorter run (Fig. 2c). Assuming that each band contains one fragment, one can estimate for the extrachromosomal elements a size of ~570 kb. Digestion with *Xho*I produced six to eight bands in the range from ~30 to ~150 kb, which add up to the same approximate size as given above (not shown). These experiments, therefore, gave results consistent with the 650-kb elements having a size between 600 and 700 kb and a homogeneous structure.

When metaphase spreads from 10B3 cells, prepared as previously described¹³, were examined in the electron microscope many extrachromosomal structures consisting of 20- to 30-nm chromatin fibres arranged in most cases in a circular or a convoluted, more complex configuration, with no free ends apparent, were observed among the chromosomes. From contour-length measurements of several of these structures, assuming a 40:1 packing ratio of DNA¹⁴, a DNA content varying between 550 and 10³ kb was estimated. Figure 3 (a and b) shows two examples of such structures. These structures are not related to the aneuploidism of 10B3 cells, because we have not observed them in any of many aneuploid cell lines analysed. Although we cannot definitely equate the structures seen in the electron microscope with the 650-kb elements, the correspondence in size strongly suggests that they are related.

In the PFGE or field-inversion gel electrophoresis (FIGE) experiments discussed above, minor slowly-moving bands hybridizable with pHD84 were frequently observed in the genomic DNA samples from the 6A3-B and 6A3-E cell lines (see, for example, asterisked bands in Fig. 1b). To investigate the nature of these components further, the effect of varying the switching regimen on their mobility during FIGE was analysed. As shown in Fig. 4, by using the pHD84 insert instead of the whole plasmid as a probe, and thus reducing the background, one minor band (a) was clearly identified in the 10B3 DNA pattern, three (b', b'' and b''') in the 6A3-B pattern, and one (e) in the 6A3-E pattern. The mobility of the 650-kb band changed markedly depending on the switching interval, and consistently paralleled that of the yeast chromosomal DNAs of similar size (not shown). By contrast, the migration of the minor bands changed only slightly with the switching interval, suggesting that they represent supercoiled circular DNAs¹².

The results presented here indicate that, in MTX-resistant human cell lines, the extrachromosomal elements containing amplified DHFR genes can be much smaller than previously thought, so as not to be recognizable by optical microscopy. In a previous electron-microscopic analysis of the minute chromosomes from a mouse MTX-resistant cell line¹³, the smallest minute was estimated, by contour length measurements, to be $\approx 5 \times 10^3$ kb. From the EtBr staining patterns one can estimate that the 650-kb band deriving from $\sim 20 \mu\text{g}$ of genomic DNA contains 50–100 ng of DNA. This is equivalent to 0.25–0.5% of the $\sim 10^{10}$ base pairs of the nuclear DNA of 10B3 cells, or to 40–80 elements per genome. If there is one DHFR gene per element, this would correspond to a significant fraction of the amplified genes detected in 10B3 cells (250–300 copies). The nature of the material in the wells containing amplified DHFR

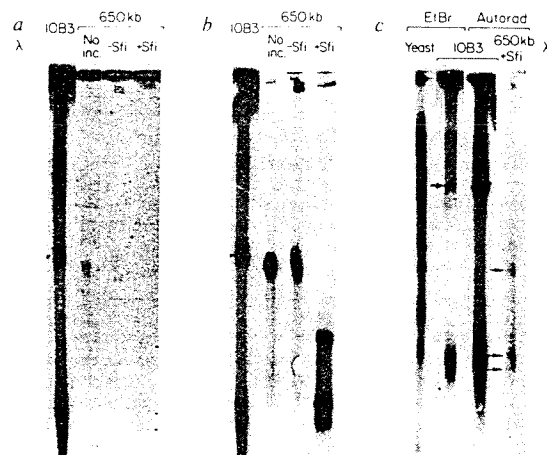


Fig. 2 *Sfi*I digestion of 650 kb elements carrying amplified DHFR genes from HeLa BU25-10B3 cells and analysis of the products by FIGE. a, EtBr-stained gel; b, DNA blot of the gel shown in a, and c, DNA blot of another gel, both probed with nick-translated DNA from the extrachromosomal elements. The gel shown in a and b was run for 19 h, with a forward-migration interval varying between 10 and 65 s and a constant 3:1 ratio between forward and reverse interval; the gel shown in c, for 18 h, with a forward-migration interval varying between 10 and 51 s.

Methods. Several samples of HeLa BU25-10B3 genomic DNA were fractionated by FIGE on a 1% SeaKem GTG agarose (FMC Bioproducts) gel as described⁸, using a modified apparatus (E. Lai *et al.*, manuscript in preparation). Agarose plugs were excised from the region corresponding to the 650-kb elements of several adjacent lanes, and extensively washed with 10 mM Tris buffer, pH 8.0, 50 mM NaCl, 1 mM EDTA, at 4°C. a and b, Two agarose-DNA slices were incubated for 3 h at 50°C with 200 units of *Sfi*I (New England Biolabs) in 0.5 ml of buffer, and then for 2 h at 50°C with proteinase K at 2 mg ml⁻¹. Two other agarose-DNA slices were treated in the same way, except for the absence of restriction enzyme. The two pairs of agarose-DNA slices, extensively washed with 0.5×TBE, and another agarose-DNA slice which had not been treated ('No inc.' lane) were embedded at the origin of a 1% SeaKem ME agarose slab, and subjected to FIGE in parallel with a sample of total genomic 10B3 DNA and with a bacteriophage λ DNA ladder (not shown). The split band in the samples incubated with or without *Sfi*I are due to mis-alignment and non-coherent migration of the two samples introduced into each well. After blotting the gel, the Zeta-Probe membrane was probed with nick-translated DNA from the extrachromosomal elements (electroeluted by FIGE). c, An agarose-DNA slice digested with *Sfi*I, as described above, was subjected to FIGE in parallel with a yeast DNA sample and with a λ ladder (not shown) for a shorter time than the samples in a and b.

genes is not known, although trapping of the submicroscopic elements by the larger chromosomal DNAs or presence of oligomers of these elements could be important.

The structure of the 650-kb elements is still uncertain. The mobility of the 650-kb elements in PFGE and FIGE responds to changes in pulse times in a similar way to that of the linear chromosomal DNAs, and differently from that of small supercoiled circular DNAs. Furthermore, it is unlikely that the 650-kb elements are large relaxed circular DNA molecules, as it is known that such molecules, like the 300–350-kb *Saccharomyces cerevisiae* K192 chromosome III DNA, are greatly retarded or even fail to enter an agarose gel (C. F. Hui, C. L. Smith, M. Mathew & C. Cantor, personal communication). Nothing, on the contrary, is known about the behaviour in PFGE or FIGE of large supercoiled DNA molecules. If the structures recognizable by E.M. are related to the 650-kb elements, as seems most likely, their apparent circularity may result from sticking of the ends of linear chromatin fibers; alternatively, the elements detec-

Table 1 Chromosome constitution of MTX-resistant human cell lines

Cell line	Complement*	No. of chromosomes per cell†	No. of minutes per cell‡	Presence of HSR*
HeLa BU25-10B3	1S(95) 2S(5)	64 ± 1 93	6(0-12)	—
VA ₂ B-6A ₃ -B	1S(97) 2S(3)	109 ± 5 203; 214	209(50-566)	—
VA ₂ B-6A ₃ -E	1S(98) 2S(2)	104 ± 7 216	206(76-535)	—
VA ₂ B-6A ₂	1S(80) 2S(20)	56 ± 2 108 ± 2	150(14-482) 220(99-452)	+(97) +(100)

A total of 18 to 63 metaphase spreads were analysed from each cell line.

* Numbers in parentheses indicate the percentage of cells in each category. In HeLa BU25-10B3, the absence of HSR has been confirmed by *in situ* hybridization experiments. 1S and 2S indicate cells with the stem-line number of chromosomes and twice that number, respectively.

† Mean ± standard deviation. Whenever only one or two metaphases were found in a given group, the individual values are reported.

‡ Mean and range.



Fig. 3 Electron micrographs of extrachromosomal elements observed in 10B3 cells. *a*, ~580 kb, ×30,000; *b*, ~710 kb, ×41,000. The DNA contents were estimated from the contour lengths of the molecules, assuming a 40:1 packing ratio of DNA in the 20- to 30-nm chromatin fiber¹⁴.

Methods. Preparations were made from cells arrested in metaphase by incubation for 5 h in Colcemid (Gibco) at a final concentration of 90 ng ml⁻¹. After harvesting, the cells were pelleted, resuspended in 0.2 vol of medium and lysed by dilution with an equal volume of 1% NP40 (pH 10), and the lysate was deposited on an electron microscope grid as previously described¹³. After air-drying, the grids were stained with 1% phosphotungstic acid and viewed in a JEOL 100C electron microscope operated at 80 kV.

ted by PFGE or FIGE may indeed be large supercoiled molecules. It is not known what relationship the minor types of DHFR gene-containing structures detected in 10B3, 6A3-B and 6A3-E cells have to the 650-kb elements.

The remarkable homogeneity in size of the 650-kb elements, which contrasts with the typical size variability of microscopically recognizable minute chromosomes, and their absence in other human MTX-resistant variants containing minute chromosomes suggests that they may arise by a mechanism distinct from that producing the latter structures. The occurrence of amplified DNA in 30-kb extrachromosomal circles of drug-

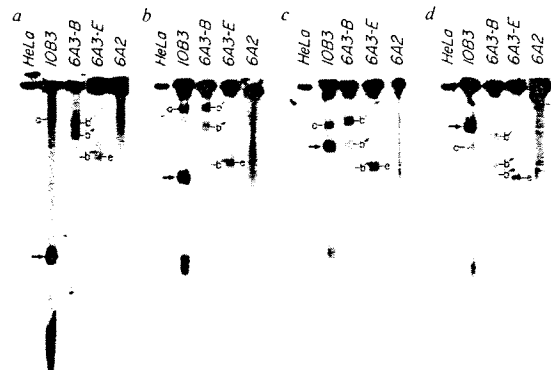


Fig. 4 Effect of changing the switching interval on the mobility in FIGE of the DHFR-gene containing extrachromosomal elements from 10B3, 6A₃-B and 6A₃-E cells. The human genomic DNA samples shown were run in FIGE using different switching regimens, as specified below; the gels were then blotted on Zeta-Probe membranes, and these were incubated with a nick-translated probe derived from the total pHd84 plasmid (*a*) or from the cDNA insert (*b*, *c* and *d*). The forward migration interval was 30-75 s in *a*, 30-80 s in *b*, 3-75 s in *c*, and 3-50 s in *d*; in all cases, a 3:1 ratio between forward and reverse intervals was used. The runs were carried out at 14°C in *a* and at 11°C in *b*, *c* and *d*.

resistant *Leishmania major* has recently been reported¹². The possibility of isolating in substantially pure form by PFGE or FIGE the 650-kb elements and the other extrachromosomal elements containing DHFR genes will make it possible to answer important questions concerning their sequence and gene organization, their expression, their capacity to replicate, and their role in the mechanism of gene amplification.

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1. Masters, J., Keeley, B., Gay, H. & Attardi, G. *Molec. cell. Biol.* 2, 498-507 (1982).
2. Morandi, C., Masters, J. N., Mottes, M. & Attardi, G. *J. molec. Biol.* 156, 583-607 (1982).
3. Stark, G. R. & Wahl, G. M. *A. Rev. Biochem.* 53, 447-491 (1984).
4. Maurer, B. J., Barker, P. E., Masters, J. N., Ruddle, F. H. & Attardi, G. *Proc. natn. Acad. Sci. U.S.A.* 81, 1484-1488 (1984).
5. Human Gene Mapping 8 (1985): Eighth International Workshop on Human Gene Mapping. *Cytogenet. Cell Genet.* 40, Nos. 1-4 (1985).

6. Schwartz, D. C. & Cantor, C. R. *Cell* 37, 67-75 (1984).
7. Carle, G. F. & Olson, M. V. *Nucleic Acids Res.* 12, 5647-5664 (1984).
8. Carle, G. F., Frank, M. & Olson, M. V. *Science* 232, 65-68 (1986).
9. Carle, G. F. & Olson, M. V. *Proc. natn. Acad. Sci. U.S.A.* 82, 3756-3760 (1985).
10. Van der Ploeg, L. H. T., Schwartz, D. C., Cantor, C. R. & Borst, P. *Cell* 37, 77-84 (1984).
11. Kemp, D. J. *et al. Nature* 315, 347-350 (1985).
12. Garvey, E. P. & Santi, D. V. *Science* 233, 535-540 (1986).
13. Hamkaio, B. A., Farnham, P. J., Johnston, R. & Schimke, R. T. *Proc. natn. Acad. Sci. U.S.A.* 82, 1126-1130 (1985).
14. Finch, J. T. & Klug, A. *Proc. natn. Acad. Sci. U.S.A.* 73, 1897-1901 (1976).

CHAPTER SEVEN ADDENDUM

**Figure 7-1. Sucrose Gradient Sedimentation of
Metaphase Chromosomes from Hela BU25-10B3.**

Figure Legends

Figure 1. Distribution of sequences hybridizable with a human DHFR cDNA probe, a human HMG-CoA reductase cDNA probe and a human rDNA probe among chromosomal structures isolated from HeLa BU25-10B3 cells and fractionated on a sucrose gradient. Sedimentation was from left to right.

METHODS

Chromosome Fragmentation. HeLa BU25-10B3 cells, which had been maintained for ~ 12 months in the presence of 1.8×10^{-4} M DL-MTX, were grown to 2×10^6 cells per 10 cm Petri dish, were treated for 24 h with 0.1 μ g/ml vinblastin sulfate, trypsinized and washed three times with 0.15 M NaCl, 0.05 M KCl, 0.001 M MgCl_2 . A sample of 0.5 ml of packed cell volume was resuspended in 20 ml of 0.075 M KCl prewarmed to 37°C and incubated for 10 min. at 37°C. The cells were pelleted and resuspended in 2.5 ml 0.075 M KCl at 37°C, and the mitotic index determined by microscopy (70 to 80%). After slow addition, under gentle swirling, of 10 ml of 50% acetic acid at room temperature and then of 12 ml of 12% (w/v) hexylene glycol in 10^{-4} M Pipes, 5×10^{-4} M CaCl_2 , the cells were broken to the extent of 75 to 85% by three consecutive passages of the suspension through a 23 gauge needle. The suspension was then made 1% in Tween 20 and loaded onto a 250 ml of 10 to 40% (w/v) sucrose in HEPICAT medium [12% (w/v) hexylene glycol, 10^{-4} M Pipes, 6×10^{-4} M CaCl_2 , 0.1% Tween 20]. The gradient was slowly brought to 700x g in an International Centrifuge Model PR-6, and centrifuged for 90 min at room temperature with a 10 min.

braking period. Twenty ml fractions were collected from the top of the gradient with a wide bore pipette and, after addition of 10 ml of 10% (w/v) sucrose in HEPICAT medium to each fraction, centrifuged in an SW27 Beckman rotor at 20 krpm for 30 min. The pellets were resuspended in 1 ml of TEN buffer (10 mM Tris buffer, pH 8.0 (25°C), 1 mM EDTA, 50 mM NaCl), pelleted by centrifugation in an Eppendorf microfuge, resuspended again in 1 ml of TEN buffer, and, after addition of 0.5% sodium dodecyl sulfate and 50 µg/ml proteinase K, incubated overnight at 37°C. After phenol extraction and ethanol precipitation, the samples were incubated for 3 h at 37°C with 50 µg/ml RNase A (preheated to 80°C for 10 min) and 20 units/ml RNase T₁ in 0.5 ml of 10 mM Tris buffer, pH 8.0, 1 mM EDTA, 100 mM NaCl. After a further phenol extraction and ethanol precipitation, the final pellets were dissolved in 10 mM Tris buffer, pH 8.0, 1 mM EDTA.

DNA transfer hybridization. Samples of the chromosome fractions were made 0.3 M in NaOH, kept at room temperature for 15 min. then cooled to 0°C for 5 min., made 1 M in ammonium acetate, pH 6.0, and transferred to a pre-wetted nitrocellulose filter with a slot blot apparatus (BRL). The filters were washed twice with 0.3 M NaCl, 0.03 M Na citrate, and baked for 2 h at 80°C in a vacuum oven. Hybridization was carried out as described, using duplicate filters with each of the following probes (1.2 to 5x10⁷ cpm), which had been nick-translated according to standard procedures to a specific activity of 4 to 8x10⁸ cpm/µg: HeLa S3 DNA (fraction reannealed to Cot 100); pHD84, a plasmid containing a human DHFR cDNA insert; λHr11, a λ Charon 4A clone containing a human rDNA insert, and pHR9, a plasmid containing a 2.1 Kb fragment of human HMG-CoA reductase cDNA (F. Preugschat and B. Wold, personal

communication). The filters were air dried and exposed for autoradiography, and the radioactive spots were then cut out and counted in a scintillation counter. The radioactivity in each sample, normalized for the DNA content of the sample on the basis of the HeLa S3 hybridization data, is expressed as a percentage of the maximum radioactivity in different fractions.

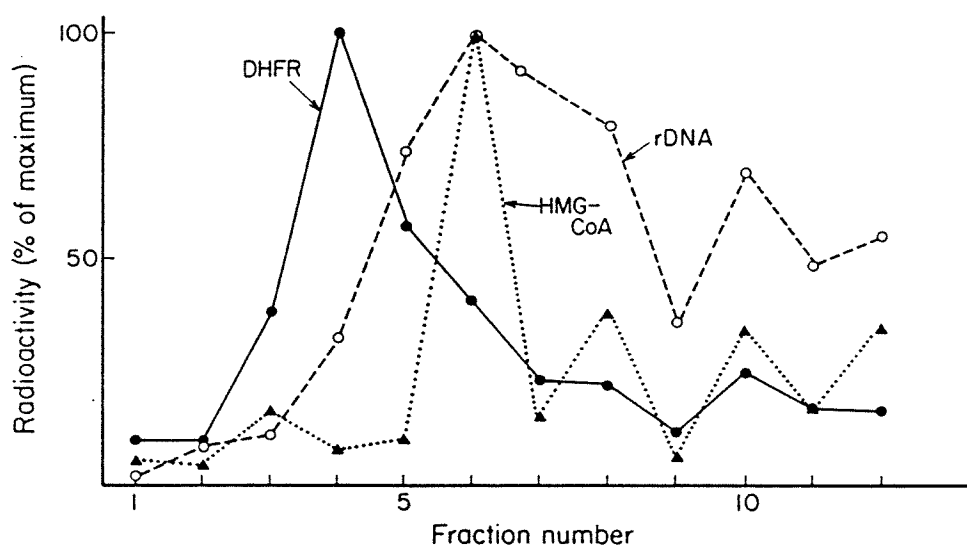


Figure 7-1. Sucrose Gradient Sedimentation of Metaphase Chromosomes from HeLa BU25-10B3.

PROPOSITION ONE

Molecular Characterization of the Hela 10B3 650 Kb Amplisome: Internal Structure and Homology with the Normal Dihydrofolate Reductase Gene Locus

Introduction

The molecular mechanism(s) producing gene amplification in mammalian cells is obscure. Numerous proposals have been put forth to explain observed data from numerous systems, including de novo amplified and drug-selected genes, both endogenous and transected, in several species (6,51-55,99). Molecular characterization of amplified DNA sequences has been hampered by the characteristically large units of amplification (amplicons) observed, some estimated to be >10,000 kb (101).

The isolation of a circular, extrachromosomal element containing amplified endogenous dihydrofolate reductase (DHFR) genes in a methotrexate (MTX)-selected human cell line lacking abnormal staining regions (ASR,HSR) and double minute chromosomes (DM), structures associated with gene amplification (7), and the presence of this element from the earliest detectable stages of gene amplification (97), suggest that such "amplisomes" (97) may represent an initial or an obligatory intermediate in the amplification of endogenous human genes. Similar molecules have since been described as containing amplified c-myc genes in the human leukemia cell line HL-60 and human neuroendocrine tumor cell line COLO320 (116), the multidrug-resistance gene MDR1 in the human carcinoma KB-V1 cell line (107), and transfected CAD genes in a PALA-selected Chinese hamster ovary cell line (76), and have been shown to be self-replicating in several of these systems (98). These findings validate these structures as

common mediators of mammalian gene amplification, at least, in vitro.

It would be informative, therefore, to examine the internal DNA structure and sequence of this element to determine if residual evidence remains of its generative mechanism from the normal DHFR gene locus on chromosome 5 and to gain insight into its replicative maintenance and regulation of gene expression. Of particular interest, are whether the element: 1) is a circularized 650 Kb DNA sequence containing one DHFR gene/element as a unit amplicon or contains multiple DHFR genes structured as tandem or inverted repeats, 2) represents a contiguous, possibly rearranged, DNA sequence from the normal DHFR gene locus or contains DNA sequences remote to the locus indicating a more extensive recombination, 3a) has unique junctional sequences representing the original chromosomal breakpoints, 3b) and whether these junctional sequences contain local organization of generative significance, 4) whether the element contains other coamplified genes and their state of expression, 5) the number and organization of replication origins contained within the element.

Proposal

The 650 Kb amplicon containing amplified endogenous DHFR genes in the human cell line Hela Bu25 10B3 should be molecularly characterized with respect to its internal DNA structure and homology to the normal DHFR gene locus.

Experimental approach

The above questions will be addressed by a) performing restriction endonuclease fragment mapping of the 650 Kb amplicon using rare-sequence cutting restriction endonucleases and comparing this map to that of the normal

DHFR gene chromosomal locus and, b) by molecular cloning of the amplisome for fine restriction endonuclease mapping, with subcloning of sequences of particular interest (such as junctional sequences) for DNA sequence and assessment of expression of coamplified genes.

Restriction endonuclease mapping. It has been shown that the 650 Kb amplisome can be isolated to homogeneity in either a linear (broken) or circular form using pulsed field gel electrophoresis (PFGE), can be subsequently digested with restriction endonucleases and can be labelled for use as a DNA probe in DNA-DNA hybridization analyses (Chapter Seven). Thus, the element can be treated in the manner of a conventional plasmid for purposes of restriction endonuclease mapping, using either the whole element or subcloned fragments as a self-probe. The expected result is a large fragment, circular map that will reveal the gross internal structure of the element, including fragments that contain the DHFR gene(s). This mapping can be further refined by fine mapping after molecular subcloning. Maximal yields of the element can be obtained using irradiated PFGE prepared DNA or SAL I restriction endonuclease digestion coupled with PFGE (97). Comparison of this map with the normal DHFR gene locus can be made by cutting parental Hela Bu25 genomic DNA with the same restriction endonucleases and probing with either whole amplisome DNA that has been radiolabelled and hybridized in the presence of excess unlabelled human repetitive DNA (Cot 0-100), or using unique sequence, subcloned, amplisomal DNA. Comparison of the restriction maps should quickly reveal whether the element represents a continuous DNA sequence encompassing the normal DHFR gene locus or has a more complex structure. Further, restriction fragments containing junctional sequences may be readily apparent as

discordant map fragments and directly subcloned for further analysis.

Molecular Cloning. The ability to isolate the amplisome to purity should facilitate its molecular cloning. It may be possible to clone the entire element in as few as two dozen overlapping cosmid clones. The cosmids can be used to further refine the restriction endonuclease map for examination of internal structure and locus comparison. The cosmids can also be used to determine if non-chromosome 5 DNA (the site of the normal DHFR gene) is present in the element by using them to screen a murine-human somatic cell hybrid library containing overlapping subsets of human chromosomes. The presence of such DNA would indicate that extensive recombination occurred either prior, during, or subsequent to the generation of the amplisome and may have mechanistic implications. These clones can also be used to determine whether other genes have been coamplified on the element and their manner of expression by using them to probe both total and polyadenylated RNAs isolated from both the HeLa Bu25 parent and its 10B3 derivative. While not all co-amplified genes may be detected in this manner, comparative levels of expression may lead to insights about the regulation of genes in highly amplified states.

Discussion

The molecular characterization of the 650 Kb element responsible for the amplification of the endogenous DHFR gene in the HeLa 10B3 cell line may give insights into its generative mechanism and would allow a comparison of similar data from genes amplified via ASRs and DMs in other systems. Further, should the HeLa 10B3 cell line progress to develop ASRs or DMs upon further growth in vitro, examination of the internal structure of this DNA should directly reveal the

evolutionary relationship, if any, between these three forms of mammalian gene amplification.

PROPOSITION TWO

Utility of the Human Cell Line Hela 10B3/650 Kb Amplisome as an Expression/Vector System to Investigate Mammalian Gene Regulation

Introduction

The study of mammalian gene expression and regulation and of chromosome structure is complex. The problem is often approached by transfection of processed genes into cells of another species in an attempt to isolate the processes and facilitate analysis. However, mammalian genes can span hundreds of kilobases of DNA, not counting possible control regions, and processes such as immunoglobulin class switch and co-ordinate gene expression may occur over similar distances. Recently, yeast artificial chromosomes (YACs) have permitted the cloning of DNA fragments approaching these sizes in yeast, but a similar, stable cloning system for mammalian cells is lacking. Thus, attempts to obtain a biologically significant understanding of these processes are severely limited by the size of the DNA sequences accommodated by current mammalian extrachromosomal cloning vectors, and may be complicated by the abnormal chromatin milieu of chromosomally transfected genes.

Recently, a new class of circular, self-replicating, extrachromosomal, transcriptionally active molecules, termed "amplisomes," have been isolated in several mammalian systems (Chapter Seven, 97, 98). These elements are active

in mammalian gene amplification and have ranged in size from 200 Kb to 750 Kb. Genes observed to be amplified by amplisomes include human dihydrofolate reductase (DHFR), c-myc and MDR1 in various human cell lines, and CAD genes in a transfected Chinese Hamster Ovary cell line (Chapter Seven, 76,116,117). While these elements have been described as unstable in some systems (98), in one, namely the 650 Kb amplisome conferring methotrexate (MTX) resistance via DHFR gene amplification in the human cell line Hela 10B3, they have remained stable under selective pressure for extended periods of time (> 70 weeks) (97). Whether this extended extrachromosomal stability is a property of this specific amplisome or of its host cell line is not clear. However, should it prove possible to manipulate these elements in vitro so as to introduce exogenous DNA sequences into them at will, and to reintroduce them into mammalian cells, then the ability to address questions of mammalian gene expression and chromatin structure over extended distances in a natural chromatin milieu would be greatly facilitated.

Proposal

An attempt should be made to transfect the Hela 10B3 650 Kb amplisome, or its derivative, into well characterized murine, hamster, chimpanzee and human cell lines, including its parent cell line Hela Bu25 and its host cell line Hela 10B3. An attempt to transfect amplisomes from other systems, particularly the 200 Kb amplisome containing CAD genes from CHO cells (76) and the 750 Kb

amplisome containing MDR1 genes from human KB cells (107), into the HeLa 10B3 cell line or its derivatives should also be made. Should it be possible to reintroduce amplisomes into cells in a stable fashion, then an attempt should be made to utilize them as cloning/expression vectors by introducing exogenous DNA sequences into them prior to transfection.

Experimental Approach

As it is unclear whether stability is a property of the 650 Kb amplisome or the HeLa 10B3 host cell line, both must be investigated. A prerequisite for use of the amplisome as a cloning vector is a partial molecular characterization of the element (see Proposition One).

Transfer of isolated amplisome or its derivatives into mammalian cell line. The 650 kb amplisome of HeLa 10B3 can be isolated to purity using pulsed field gel electrophoresis (PFGE) in both a linear (broken) and circular form (BM thesis) or yields maximized by irradiating or Sal I endonuclease digesting PFGE prepared DNA prior to PFGE (97). Attempts to reintroduce the element back into mammalian cells may be problematical due to its large size. Exogenous DNA has been introduced into mammalian cells by the CaPO_4 precipitation technique, direct nuclear injection, electroporation, artificial liposome fusion and microsphere penetration. The large size of the

amplisome mitigates against electroporation and microsphere penetration, and may exclude nuclear injection (secondary to shear effects). Efforts, then, would be directed towards the CaPO_4 precipitation technique, liposome fusion, and nuclear injection of both linear and circular forms of the element. Transfected cells would be selected by MTX resistance conferred by the DHFR gene contained in the amplisome. It is suspected that utilizing the circular form of the amplisome would have the greatest chance of success due to its smaller Stokes radius (reducing shear breakage), as well as decreasing the probability of concatenate formation and chromosomal insertion.

Transfection will be attempted in well characterized cell lines of several species, including Hela 10B3 cells depleted of amplisomes by extended growth in nonselective conditions (97), to determine compatible host ranges. Successful transfection and extrachromosomal maintenance should result in high level MTX resistance in a single step if multiple copies of the element are transferred, or rapid increases in resistance with selection, a hallmark of extrachromosomal gene amplification (99,103), if fewer copies are transferred. Recovery of the amplisome using PFGE confirms successful extrachromosomal transfer. Extrachromosomal location without chromosomal insertion of some of the elements can be confirmed by the rapid loss of the amplified genes in the absence of selection (13) and by *in situ* hybridization.

An attempt should also be made to transfect after reducing the size of the element by self ligation in limiting dilution after partial restriction endonuclease

digestion. Proper choice of enzymes after partial molecular characterization of the amplisome will allow retention of the selectable DHFR gene. Several enzymes should be used in an attempt to avoid deleting essential sequences such as replication origins. It is probable that the smaller the amplisome is, the easier transfection would be.

Successful transfection would allow the introduction of exogenous DNA sequences into the element, including additional drug resistance genes, and transform the amplisome into a cloning vector.

Control of amplisome copy number per cell could be controlled by selective pressure (97).

Preliminary host range experiments can be carried out using somatic cell fusion of the Hela 10B3 cell line with candidate cells.

The effect, if any, of cell cycle on successful transfection could be assessed using synchronized cell cultures.

Successful transfection of such large fragments of DNA is not a forgone conclusion, particularly when introducing exogenous sequences into the amplisome, as ligation kinetics may be limiting. However, successful manipulations of large, Inc P2 plasmids (>350 Kb) from *Pseudomonas* sp. have been reported and imply that molecules of this size are at least partially amenable to in vitro techniques.

Transfection of Hela 10B3 cell line and amplisome-depleted derivatives by

exogenous amplisomes. To ascertain if amplisome stability is a property of the HeLa 10B3 cell line, drug-selectable amplisomes from other systems, the 200 Kb CAD amplisome and the 750 Kb MDR1 amplisome, will be transfected into HeLa 10B3 and examined for stability as above. As both amplisomes have previously been reported as unstable in their respective host cell lines, transfection with stability would indicate that stability is a property of the HeLa 10B3 cell line. Instability would indicate that these amplisomes are inherently unstable and imply that stability is a property of the 650 Kb DHFR amplisome.

It is possible that stability is the result of a unique interaction between HeLa 10B3 and the DHFR amplisome. This can be addressed by transfecting the amplisome into HeLa 10B3 derivatives that have been depleted of the element by extended growth in nonselective conditions. Stability of the amplisome in HeLa 10B3, but not in other cell lines, would indicate such an interaction.

Discussion

The extrachromosomal stability of the 650 Kb amplisome in HeLa 10B3 implies that only technical manipulation remains before such elements can be used to study large DNA fragments retaining all associated genes and regulatory sequences in mammalian cells. The ability to control copy number via selective pressure allows investigation of gene dosage effects, of particular relevance to the study of oncogenes and tumorigenesis. Other questions that can be

addressed will be the minimal sequence requirements for a functional mammalian centromere and the possible existence of remote gene expression control sequences, among others.

PROPOSITION THREE

**The Isolation and Characterization of Submicroscopic
Extrachromosomal DNA Elements from Human Leukemias**



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NEW REQUEST

☐

RENEWAL REQUEST

APPLICATION FOR RESEARCH GRANT

Medical Advisory Board

Children's Leukemia Foundation of Michigan

Date: Jan. 5, 1988

Application is made for a grant in the amount of \$45,025 for the period from July 1, 1988 through June 30, 1990 inclusive, for the support of research on the following subjects:

The Isolation And Characterization Of Submicroscopic Extrachromosomal DNA Elements From Human Leukemias. This work will analyze how these Elements Contribute to transformation and progression

(Give only brief descriptive title of research; details on attached pages)

David I. Smith Ph.D. Assistant Professor
(Name of Principal Investigator) (Degree) (Title)

3136 Scott Hall, 540 E. Canfield, Detroit, Michigan 48201
(Institutional Address of Principal Investigator)

Wayne State University School of Medicine
(Institution where work is being done)

3136 Scott Hall, 540 E. Canfield, Detroit, Michigan 48201
(Address)

Shirley Walkowski, Associate Director, Office of Research and Sponsored Prog.
(Give name and title of responsible institutional financial officer to whom funds should be sent and who will keep full account of disbursements.)

Approved by Executive Officer of Institution under whose auspices the research is to be carried out.

David I. Smith
(Signature of Principal Investigator)

Wayne State University, School of Medicine
(Name of Institution)

(Signature)

(Title)



Children's Leukemia Foundation of Michigan

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CURRICULUM VITAE -CO-INVESTIGATOR (if any)

NAME: Maurer Barry James B.S. B.S.E.(Ph.D.)*
 Last First Middle Degree(s) *Thesis Defense,
 Caltech, Mar. 1988

Birthdate: 9/27/ 1956 Birthplace: Michigan Citizenship: U.S. Other: USA Sex: Male
 Mo. Day Year State or Country (specify) (M-F)

EDUCATION (Begin with entry into college)

Name & location of college/university	Dates Attended		Degree, if any (month & yr. received)	Major Dept.	Major Field
	From Month Year	To Month Year			
University of Michigan, Ann Arbor, Michigan	9/74	5/80	B.S.E. B.S.	Chem. Eng Chemistry	Chem. Eng Chemistry
California Institute of Technology	10/80	8/86* (L.O.A. 9/87-3/88)	Ph.D. (Cand.) Thesis completed Defense 3/88	Chem.	Chemical Eng.
School of Medicine Wayne State University	8/86	Present	Medical Student	Medicine	Medicine

POSITIONS HELD (State with first position held following baccalaureate and give consecutive record to date)

Department	Rank	Institution	Supervisor	Full/Part Time	Dates	
					From	To
Chemistry (Res.)	Grad. Stud. Asst, Teach.	California Inst. Tech.	G. Attardi	Full	10/80	8/86
Molecular Biology and Genetics	Med. Stud. Res. Asst.	Wayne State Univ.	O.J. Miller D.I. Smith	Part time Part time	5/87 9/87	8/87 present

List academic and professional honors including all scholarship, traineeship or fellowship awards. Give dates and source of awards, i.e., NIH, NSF, etc.
 NIH NRSA Predoctoral Traineeship (1980-1986); Fellow, Summer Science Research Program for Medical Student, March of Dimes Birth Defect Foundation, 1987

List names of selected professional societies and related organizations in which membership has been held within the last two years. Give dates.
 McCombie, R.W., Hansen, J.B., Zylstra, G.J., Maurer, B.J. and Olsen R.H.: Pseudomonas Streptomycin Resistance Transposon Associated with R-Plasmid Mobilization J. Bacteriol 155 40-48 (1983)

NEW:

X



Children's Leukemia Foundation of Michigan

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RENEWAL:

Please submit 25 copies of this
research abstract - DO NOT STAPLE.
Clip copies together and enclose
with original grant application

RESEARCH ABSTRACT

PRINCIPAL INVESTIGATOR'S NAME Smith David I.
(Last) (First) (Middle)

INSTITUTION WHERE RESEARCH WILL BE DONE Wayne State University School of Medicine

ADDRESS OF INSTITUTION 540 E. Canfield, Detroit, Michigan 48201

COMPLETE TITLE OF PROJECT: Isolation And Characterization Of Submicroscopic
Extrachromosomal DNA Elements From Human Leukemias

ABSTRACT

(250 words or less)

The molecular mechanisms leading to the amplification of drug-resistance genes and oncogenes in tumors are only partially understood. Recently, one of us has shown that amplified dihydrofolate reductase genes in several methotrexate-resistant human cell lines are located in submicroscopic, extrachromosomal elements and can be purified to homogeneity. We propose to examine drug-resistant human leukemias to determine if these elements constitute a mechanisms for gene amplification in human leukemias in vivo. Fifty to one hundred drug-resistant leukemias will be surveyed using field inversion gel electrophoresis and probed for the amplification of appropriate resistance genes including DHFR and P-glycoprotein, and leukemia-associated oncogenes (c-myc, v-abl, v-myb, and H-ras). Further, the ability to separate intact circular DNA molecules from random linear DNA molecules using our gel system permits the isolation, cloning, and molecular characterization of novel, extrachromosomally amplified DNA sequences of presumed biological relevance to drug-resistance, transformation, and malignant progression. The existence and characterization of such extrachromosomal elements in human leukemias would lead to a greater understanding of the processes of gene amplification and to possible insights into the development and clinical treatment of chemotherapeutic agent-resistant human cancers.

Date prepared: Jan. 5, 1988



Children's Leukemia Foundation of Michigan
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Investigator's Name: David I. Smith, Ph.D.

Institution: Wayne State University-Medicine

Year Grant to begin: July, 1988

OUTLINE OF PROPOSED RESEARCH

I. Aims of research program (250 words or less)

The principal aim of this proposal is to determine if genes associated with drug-resistance and transformation of drug-resistant human leukemias are amplified as submicroscopic, extrachromosomal elements (SECE) in situ. Our specific aims are:

- 1) Fractionating the extrachromosomal DNA complement of 50-100 drug resistant (relapsed) human leukemias using one-dimensional and two-dimensional field inversion gel electrophoresis.
- 2) Probing the fractionated extrachromosomal DNA by the DNA-DNA hybridization technique for the presence of known drug-resistance genes; (dihydrofolate reductase (resistance to methotrexate), P-glycoprotein (MDR)), and leukemia associated oncogenes (c-myc, v-abl, v-sis, v-myb, and H-ras)), as well as for the presence of other discretely amplified sequences of presumed biological significance.
- 3) Cloning the detected SECE for molecular characterization and for use in probing leukemia RNA's and/or cDNA libraries.

These experiments will determine if SECE exist and constitute an operative mechanism of gene amplification in leukemias in vivo, and may lead to the isolation of previously unknown DNA sequences with biological relevance to the drug-resistance, transformed, and malignant phenotypes of human leukemias. Further, we believe these investigations will lead to a greater understanding of mammalian gene amplification, in general, and to possible insights into the development and clinical treatment of chemotherapeutic agent-resistant human cancers.



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Investigators Name: David I. Smith, Ph.D.Institution: Wayne State University-MedicineYear Grant to begin: July, 1988

II. Previous work done by applicant (one page maximum)

Barry Maurer has worked extensively on the characterization of the chromosomal changes accompanying DHFR gene amplification-mediated methotrexate (MTX) resistance in human cell lines and on the isolation and characterization of novel submicroscopic, extrachromosomal elements containing amplified DHFR genes from these cell lines. Recently, he has shown that most of the amplified DHFR genes in an MTX-resistant cell line with few optically visible minute chromosomes reside in structures sedimenting more slowly than normal chromosomes in a sucrose gradient. These structures that can be resolved on orthogonal pulsed field gradient gel electrophoresis (OPHAGE) or field inversion gel electrophoresis (FIGE) as homogeneous elements of about 650 kB in size and behave as linear molecules. In several MTX-resistant cell lines, other DHFR gene-containing elements of at least 450kB in size have been observed and these behave as circular molecules. These submicroscopic elements are the first amplified mammalian gene sequences to be purified to homogeneity.

One aspect of this application is the cloning of the extrachromosomal elements found in the nuclei from leukemic patients. This laboratory is ideally suited for this work due to their extensive experience working with the construction of genomic libraries. We routinely construct genomic libraries using both lambda and cosmid cloning vectors. In addition, the P.I. has also had extensive experience with the construction and screening of cDNA libraries.



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Investigator: David I. Smith, Ph.D.

Institution: Wayne State University-Medicine

Year Grant to begin: July, 1988

- III. Previous work done by other Investigators on the same or related problems. (Cite no more than five, recent and pertinent references; one page.)

Selective gene amplification has been observed in drug-resistant mammalian cell lines and in tumors or cell lines derived from them and is generally accompanied by optically visible chromosome abnormalities. These abnormalities include double minute chromosomes (acentromeric, self-replicating, extrachromosomal elements), chromosomes containing an expanded homogeneously staining region (HSR), and small chromatin bodies (1). Both minute and HSR chromosomes have been shown to contain the amplified genes responsible for resistance in drug-selected cell lines. HSR's (and by inference, double minutes) have also been shown to contain amplified oncogene sequences in human tumors, including leukemias, and in tumor cell lines (2,3). From the estimated size of HSR's and gene copy number, it is clear that the unit of amplification (amplicon) is much larger than the amplified gene itself, ranging from approximately 250 kb to over 3000 kb (4). It has been shown that at least some of the amplicons in HSR's can be arranged tandemly or as inverted repeats. The co-amplification of genes unrelated to the selected phenotype has also been reported (5). The above observations have been obtained by chromosome walking on HSR DNA cloned into cosmids. The inability to purify amplicon DNA in its native configuration has complicated these investigations. The generative mechanisms of gene amplification in mammalian cells is not yet known. Suggested models to explain the phenomenon include unequal sister chromatid exchange, chromosome fragmentation, and onion skin over-replication. The relationship, if any, between minute and HSR chromosomes is also not clear.

References

- (1) Schmike, R.T. (ed.). GENE AMPLIFICATION. Cold Spring Harbor Laboratory, New York, 1983.
- (2) Shiloh, Y., Shipley, J., Brodeur, G.M., Bruns, G., Korf, B., Donlon, T., Schreck, R.R., Seeger, R., Sakai, K., Latt, S.A. PNAS 82U:3761-3765, 1985.
- (3) Start, D.R., Wahl, G.M. ANN. REV. BIOCHEM. 53:447-491, 1984.
- (4) Bostock, C.J. and Clark, E.M. CELL 19:709-15, 1980.
- (5) VanDer Bliek, A.M.; VanDer Velde-Koerts, T., Lung, V., Borse, P. MOL CELL BIOL 6:1761-1678, 1986.



Children's Leukemia Foundation of Michigan

SERVING ADULTS AND CHILDREN

Investigators Name: David I. Smith, Ph.D.Institution: Wayne State University-MedicineYear Grant to begin: July, 1988

IV. Contemplated Method of Approach (1 to 3 pages: for additional pages, include Investigator and Institution name in upper right corner; to be numbered etc.)

Our aim is to determine if genes associated with the drug-resistance and transformed phenotypes of drug-resistant (relapsed) human leukemias are amplified as submicroscopic, extrachromosomal elements in situ. The problem will be approached as follows:

- 1) Fractionating the extrachromosomal DNA complement of 50-100 drug resistant human leukemias using one-dimensional and two-dimensional field inversion gel electrophoresis.

White blood cells from peripheral blood or bone marrow samples are isolated on ficoll-hypaque gradients, resuspended in agarose and extensively digested with proteinase K. This procedure results in very high molecular weight DNA embedded in agarose. The DNA is then separated on agarose gels using FIGE, a system using timed voltage-pulses (ramp) of alternating polarities. FIGE allows discrete, extrachromosomal DNA populations plus small, randomly broken fragments to enter a gel while excluding very high (greater than 5000 kB) molecular weight chromosomal DNA. Recently, one of us (Barry Maurer, unpublished observations) has demonstrated that the migration properties of circular DNA (supercoiled and relaxed) respond differently than linear DNA to FIGE running conditions: supercoiled plasmids migrate independantly of ramp conditions, while relaxed plasmids migrate more slowly than linear molecules under longer ramp conditions. These properties permit the separation of linear (randomly broken) molecules, from circular (discrete) molecules using a novel two dimensional FIE approach: a ramp condition (10 sec-48 sec, 300 V) allowing circular and small (less than 2000 kB) linear molecules to enter the gel is applied in the first dimension. Electrophoresis under longer ramp conditions (48 sec-80 sec, 300 V) is then applied in the second dimension. The second ramp condition allows the rapid migration in the gel of linear molecules while preferentially retarding circular molecules thereby effecting their separation.

David I. Smith, Ph.D.
Wayne State University

2) Probing the fractionated extrachromosomal DNA by the DNA-DNA hybridization technique for the presence of known drug-resistance genes (DHFR, P-glycoprotein) and leukemia associated oncogenes, as well as for the presence of other discretely amplified sequences of presumed biological significance using repetitive-sequence DNA probes.

Fractionated DNA will be transferred to Zeta Probe Nylon membranes and probed by Southern blot analysis using nick-translated, radiolabeled sequences of appropriate drug-resistance genes (DHFR, p-glycoprotein) as well as leukemia associated oncogenes (c-myc, v-abl, v-sis, v-myb, and H-ras). Additional oncogene sequences may also be tested. Circular SECE which do not contain the above sequences will be detected using two dimensional FIGE separations probed with radio-labeled human repetitive-sequence DNA. The large size of SECE makes it highly likely that they will contain repeat sequences in multiple copy number, and these will be detected with our repetitive sequence probes.

3) Cloning the detected SECE for molecular characterization and for use in probing leukemia RNA's and/or cDNA libraries.

SECE found will be isolated by gel elution or by FIGE gels run in low melting temperature agarose and subcloned into convenient plasmid and cosmid vectors for in situ hybridization analysis of their chromosomal origin and for characterization of their molecular organization. Subcloned DNA will also be used to probe RNA's from leukemia cells (Northern blot analysis) to determine gene expression. Subcloned DNA will then be used to probe cDNA libraries constructed from the isolated RNA if we detect hybridization with our Northern analysis.

Tumors (peripheral blood samples collected by venipuncture and bone marrow aspirates) will be collected by contacts at the Detroit Medical Center (Dr. Ravindranath, Childrens Hospital of Michigan and Dr. Kharanes, Harper Hospital) and also applied for through the Pediatric Oncology Group. Preliminary results indicate that samples may be stored for up to 72 hours on wet ice. Controls will be the leukocytes of peripheral blood and bone marrows of non-leukemic human subjects.



Children's Leukemia Foundation of Michigan

SERVING ADULTS AND CHILDREN

Investigators Name: David I. Smith, Ph.D.Institution: Wayne State University-MedicineYear Grant to begin: July, 1988

V. Estimated duration (250 words or less)

The estimated duration of this proposal is 24 months. In the first 12 months we will analyze the DNA from 50-100 patients and begin probing circular and linear molecules purified by 2 dimensional FIGE with our battery of cloned probes. The second 12 months will be occupied with constructing libraries from isolated extrachromosomal elements, isolating unique sequence hybridization probes from recombinants, and doing Northern blot analysis with these probes to RNA isolated from leukemic patients.

VI. If the Principal Investigator intends to be absent for a period of more than 30 days during the grant year, please state below the dates of the leave, the reasons for the leave and the intentions of the investigator for continuing the research during the absence. (If not applicable, please so state below).

Not applicable.

OUTLINE OF PROPOSED BUDGET

ITEM

A. Personnel (itemize)

NAME	DEGREE (indicate if none)	% of time on project	BASE SALARY	FICA	*RETIRE- MENT	OTHER FRINGE BENEFITS	SUB- TOTAL	TOTAL AMOUNTS \$
D.I. Smith	Ph.D.	P.I., 10%	-0-	-0-	-0-	-0-	-0-	-0-
B. Maurer	Ph.D.	co-P.I., 20%	-0-	-0-	-0-	-0-	-0-	-0-
To be named	M.S.	50%	\$10,000	\$751	-0-	\$1249	\$12,000	\$12,000 per year
B. Equipment (Not to exceed \$3,500)								
Field Inversion Gel Electrophoresis Equipment								
Gel Boxes: \$500								
Power Supply: \$1500								
C. Supplies (itemize major purchases)								
DNA Modifying Enzymes \$2,000								
Proteinase K enzyme \$1,500								
Chemicals \$1,500								
D. Other Expenses (itemize)								
Radioisotopes \$1,200								
X-Ray, photographs \$1,500								
DNA hybridization membranes \$750								
Miscellaneous \$500								
Total: \$3125								
E. Estimated number of hours Principal Investigator intends to spend personally on this project per week: _____								
GRAND TOTAL:--\$								\$3125 first year
								\$8,950 per year
								45,025 for 2 years



Children's Leukemia Foundation of Michigan

SERVING ADULTS AND CHILDREN

19022 West Ten Mile Road • Southfield, Michigan 48075-2498 • (313) 353-8222



RENEWAL REQUEST

APPLICATION FOR RENEWAL GRANT

Medical Advisory Board

Children's Leukemia Foundation of Michigan

Date: Jan. 31, 1989

Application is made for a grant in the amount of \$ 20,950
for the period from July 1, 1989 through June 30, 1990 inclusive,
for the support of research on the following subjects:

The Isolation And Characterization Of Submicroscopic Extrachromosomal
DNA Elements From Human Leukemias. This work will analyze how these
elements contribute to transformation and progression.

(Give only brief descriptive title of research; details on attached pages)

David I Smith Ph.D. Assistant Professor
(Name of Principal Investigator) (Degree) (Title)

3136 Scott Hall, 540 East Canfield, Detroit, Michigan 48201
(Institutional Address of Principal Investigator)

Wayne State University School of Medicine

(Institution where work is being done)

3136 Scott Hall, 540 East Canfield, Detroit, Michigan 48201

(Address)

Shirley Walkowski, Associate Director, Office of Research and Sponsored Programs
(Give name and title of responsible institutional financial officer to
whom funds should be sent and who will keep full account of disbursements.)

Approved by Executive Officer of
Institution under whose auspices
the research is to be carried out.

David I Smith
(Signature of Principal Investigator)

Wayne State University, School of Medicine
(Name of Institution)

(Signature)

(Title)

Principal Investigator's Name: David I Smith, Ph.D.
 Institution: Wayne State University, School of Medicine
 Period of Grant: July 1, 1989 - June 30, 1990

PROGRESS REPORT

DESCRIBE PROGRESS TOWARD AIMS DESCRIBED IN ORIGINAL APPLICATION.
 MAXIMUM TWO (2) PAGES - (FRONT AND BACK OF THIS FORM):

It has been shown that amplified dihydrofolate reductase genes in several methotrexate-resistant human cell lines (HeLa, VA2-B) are located in submicroscopic, extrachromosomal elements (SECE) and can be purified to homogeneity using the FIGE gel system.¹ Further, it is known that amplification of the P-glycoprotein gene (multidrug resistance locus, MDR1, resistance to vinblastine, adriamycin, colchicine) confers drug resistance to the human leukemic lymphoblastic cell line (CEM) (ATCC, CL119)² and that early passages of HL-60 human promyelocytic leukemia cells contain amplified MYC oncogenes on submicroscopic, circular, extrachromosomal molecules.³ The significance of these in vitro studies to the processes of drug resistance and transformation in human leukemias await the demonstration that such SECE, containing drug resistance and oncogeneic genes, can be found in human tumors, in vivo. It is this demonstration that the current study addresses.

In our initial application, we proposed using the FIGE system to survey 50-100 drug resistant (relapsed) leukemias for SECE amplified resistance genes (DHFR, MDR1) and leukemia associated oncogenes such as MYC, ABL, MYB, HRAS, SIS, and KRAS. Demonstration of SECE, in vivo, would lead to their molecular cloning and characterization and would validate the utility of in vitro systems in addressing this phenomena. After CLFM funding commenced on 8/8/88, a job search to fill a half-time research associateship began. Following initial difficulties, we secured the technical assistance of Patricia Green (B.S.) and have trained her in leukemia sample preparation and FIGE techniques.

In the five funded months to date, we have surveyed 18 relapsed human leukemia samples, (HLS): 17 ALL tumors (including 3 T-cell and 1 T-cell (NK)), and one AML tumor. The samples included 16 pediatric tumors obtained from Dr. Ravidranath of Childrens Hospital of Michigan and 2 adult tumors from Dr. Kharanes, Harper Hospital. Among HLS 1-18, we have found 2 ALL tumors which are candidates to contain SECE containing sequences homologous to the probes used: HLS 9 (MDR1) and HLS 10 (MYB). Both samples gave signals at the limits of detection. To amplify signal strength, we are preparing to make use of the Polymerase Chain Reaction (PCR) sequence amplification technique⁴ on fractions of FIGE separated and eluted DNA's from these 2 samples. The PCR technique utilizes specific oligonucleotide primers and the TaqI polymerase to amplify selected nucleotide sequences 10^4 - 10^5 fold. Sensitivity using PCR should increase detection limits to 1 SECE in 10^3 to 10^4 cells, with simultaneous amplification of unique sequences of human Cytochrome C Oxidase gene serving as an internal control of DNA quantitation. The WSU Department of Molecular Biology and Genetics has recently acquired an automated PCR processor and oligonucleotide synthesizer to facilitate analyses using this approach.

We will continue to survey new tumor samples as they become available and will begin molecular cloning of the HLS 9 and HLS 10 SECEs upon their confirmation.

PROGRESS REPORT:P.I.NAME: David I Smith, Ph.D.

The recent suggestion that SECE are the precursors of double minute (DM) and HSR chromosomes⁵ make their demonstration in tumors, in vivo, particularly important and their existence would constitute a previously unsuspected mechanism of tumor progression in leukemias.

Preliminary results:

<u>HLS</u>	<u>DHFR</u>	<u>MDR1</u>	<u>MYC</u>	<u>HRAS</u>	<u>SIS</u>	<u>MYB</u>	<u>ABL</u>	<u>KRAS</u>
1	-	-	-	-	-	-		
2	-	-	-	-	-	-		
3	-	-	-	-	-	-		
4	-	-	-	-	ND	ND		
5	-	-	-	-	-	-		
6	-	-	-		-	-		
7	-	-	-		-	-		
8	ND	-	-		-	-		
9	-	(+)	-		-	-		
10	-	-	-		-	(+)		
11	-	-	-		-	-		
12	-	-	-		-	ND		
13	-	-	-		-	-		
14	-	-	-		-	-		
15	-	-	-		-	-		
16	-	-	-		-	-		
17	-	-	-		-	-		
18	-	-	-		-	-		

Survey of HLS 1-18 with ABL, HRAS and KRAS in progress.

(+)= SECE present, provisional; - = SECE not present at detection limits;
ND = not done

We thank the CLFM for their support.

1. Maurer, et.al. Nature 327: 434-437, 1987.
2. Shen, et.al. Science 232: 643-645, 1986.
3. Von Hoff, et.al. Proc. Natl. Acad. Sci. USA 85: 4804-4808, 1988.
4. Saiki, et. al. Science 230: 1350-1354, 1985.
5. Carroll, et. al. Mol. Cell. Biol. 8: 1525-1533, 1988.

OUTLINE OF PROPOSED BUDGET

ITEM

A. Personnel (itemize)

NAME	DEGREE (Indicate if none)	% of time on project	BASE SALARY	FICA	*RETIRE- MENT	OTHER FRINGE BENEFITS	SUB- TOTAL	TOTAL AMOUNTS \$
D.J. Smith	Ph.D.	P.I., 10%	-0-	-0-	-0-	-0-	-0-	-0-
B. Maurer	Ph.D.	co-P.I. 20%	-0-	-0-	-0-	-0-	-0-	-0-
P. Green	B.S.	50%	\$10,000	\$751	-0-	\$1249	\$12,000	\$12,000
B. Equipment (Not to exceed \$3,500)								
None								
C. Supplies (itemize major purchases)								
DNA modifying enzymes			\$1250					\$1250
Proteinase K enzyme			\$1000					\$ 750
D. Other Expenses (itemize)								\$ 500
Chemicals			\$1200					
Radioisotopes			\$1500					
X-ray photography			\$1500					
E. Estimated number of hours Principal Investigator intends to spend personally on this project per week:			5					
GRAND TOTAL: \$							20,950	20,950

PROPOSITION FOUR

Detection of Submicroscopic 15q11-13 Deletions Associated with Angelman Syndrome: Locus Definition and Diagnostic Markers

Introduction

Angelman (Happy Puppet) Syndrome (AS) is a rare congenital disorder whose clinical diagnostic criteria include a puppet-like, broadly based, jerky gait with flexed arms in abduction, a happy disposition with paroxysms of laughter, severe mental retardation, profound speech delay, a characteristic electroencephalogram pattern and seizure. Features also associated include light irides and skin pigmentation, facial dysmorphism including a prominent, pointed jaw, wide mouth, macrostomia with tongue protrusion, microcephaly and brachycephaly (126-128,132-134). The syndrome has been associated with karyotypically visible deletion of the maternal 15q12 region in at least some cases (124,125,129,130), a region also associated with the phenotypically distinct Prader-Labhart-Willi Syndrome (PLWS) (134) .

PLWS is a common congenital disorder (1/10,000–1/30,000) accounting for, perhaps, 1% of all mentally retarded persons. Clinical diagnostic criteria include infantile hypotonia, early childhood obesity with hyperphagia and food-seeking behavior, psychomotor developmental delay with mental retardation (IQ 20-80), hypogonadism, small hands and feet and short stature. Associated features include fair hair, eyes and skin, sun sensitivity and facial dysmorphism (117-120). PLWS has been associated with karyotypically visible deletions of the paternal 15q12 region (60%) and other chromosome 15 abnormalities (117,120,122,134). Recent evidence suggests that the remaining cases of PLWS may result from maternal heterodisomal inheritance of chromosome 15, the first

evidence of heterodisomal-induced disease reported in humans (121), although absence of small deletions of both maternal chromosome 15q12 regions have not been ruled out in these cases.

Strict association of both disorders with 15q12 is, however, problematical as it is not observed in all patients and, particularly, as this region has been reported to be karyotypically variable in normal individuals (125). Further, patients are often "found" to have 15q12 deletions upon rescreening of chromosomes originally reported as normal (125), and in the absence of parental karyotypes for comparison.

The assignment of parental chromosomes carrying the deletions is also confused by the recently raised possibility of heterodisomal inheritance. The assignment of parental chromosome deletion is most often based upon karyotypic appearance and the assumption of biparental inheritance, seldom being verified using DNA restriction fragment length polymorphisms (RFLP). If heterodisomal inheritance is common in these two disorders, then proper karyotypic assignment of parental chromosomes is in doubt and current statistics may be grossly inaccurate.

The clinical diagnosis of both disorders also suffers from a lack of uniform diagnostic criteria and of rigorous precision in their application: a medical syndrome, by definition, need only meet a variable number of, often subjective, inclusionary criteria for the diagnosis to be made. Possible variation in phenotypical expression of a common genotype may lead to further diagnostic difficulties.

It is necessary, therefore, that the gene(s) responsible for AS and PLWS be molecularly characterized so that an accurate diagnosis and prognosis can be made and to distinguish them from other possibly phenotypically similar, but

genetically distinct, disorders.

The molecular characterization of the PLWS locus, presumptively at 15q12, is currently underway using DNA sequence markers from the 15q11-13 region (122,123,131).

It is of interest that there is a partial phenotypic overlap between AS and PLWS. Both have a statistically significant incidence of fair pigmentation (about 2/3). In PLWS this is highly correlated with a visible 15q12 deletion ($P < .001-.05$) (119,120). This suggests that the AS and PLWS loci are separated by pigmentation loci which are variably affected in their respective patients, resulting in disparate phenotypes which, however, share fair pigmentation as a common feature.

The neurology department at the Children's Hospital of Michigan (CHM) provides health care for at least four confirmed and three suspected cases of AS, all reported to be negative for deletion of the 15q12 region. It is proposed to examine the DNA of these patients using rare-cutting restriction endonuclease and pulsed field gel electrophoresis (PFGE) of the large RFLPs to ascertain if currently available DNA probes can detect submicroscopic deletions that may assist in delimiting the AS locus and that may serve as diagnostic criteria in the evaluation of future AS patients. As the number of AS patients diagnosed to date is small, possibly < 150 , even a limited patient series may prove extremely useful in reducing candidate DNA regions to a minimum.

Proposal

The DNA of confirmed and suspected AS patients at CHM and their parents will be examined using RLFP analysis using rare-cutting restriction endonucleases and currently available 15q11-13 DNA probes in an attempt to

detect small DNA deletions/rearrangements in this region that may delimit the AS locus. Should such a region be detected, it will be cloned and molecularly characterized in both affected and normal individuals and an attempt made to link the region to the PLWS region. The RFLP analysis may also determine if heterodisomal inheritance has occurred in any of these patients. The goal is characterization of the AS locus and generation of diagnostic DNA probes.

Experimental Approach

To date, DNA from peripheral leukocytes obtained by venipuncture from three complete and two incomplete family series (complete = patient and both parents) from four confirmed and one suspected AS patients at CHM has been prepared for PFGE RFLP analysis. Current work on the PLWS locus has generated numerous unique sequence DNA probes specific for the 15q11-13 region (122,131). We are currently using five such probes: D15S9, S10, S12, S13 and S14. At least one of these probes, D15S10, is associated with deletion in PLWS with 15q12 karyotypically visible deletions (122,131) .

Rare-cutting restriction endonucleases which give RFLPs with these probes in the 200 Kb – 1,500 Kb size range are currently being employed. These include Rsr II, Nru I, Mlu I and Sst II, which were found to yield suitable fragments using leukocyte DNA from healthy individuals and which appear to be minimally methylation sensitive at these sites.

It is hoped that the use of RFLPs > 1,000 Kb to span large regions will offset the formal lack of linkage of the DNA probes used. The use of such large fragments does, however, reduce the size of the deletion that can be reliably detected. Therefore, smaller RFLPs in the 200 Kb – 500 Kb range are also being examined.

The DNA has been transferred to nylon membranes (Nytran, Zetaprobe) after PFGE to facilitate this analysis since the same membranes can be reprobbed at least five times in our hands using probes p³²-labelled by the random priming or nick-translation methods. As more probes become available, these membranes can be conveniently reprobbed. The stability of PFGE DNA samples for greater than two years will enable us to use other restriction endonucleases as the chromosomal order of current and new probes are defined and the maximally efficient RFLPs determined.

Further, leukocytes from four of the AS patients have been frozen for storage so that transformation to permanent cell line can be performed should deletions be evidenced. Permanent cell lines from these patients will allow molecular cloning of their affected regions and provide the potential for gene expression studies.

The examination of large RFLPs may directly allow evaluation of heterodisomal inheritance, if present. If not, this will be evaluated by conventional methods (121).

Discussion

AS is a currently rare (underdiagnosed?), congenital, mental retardation disorder that appears to be closely linked to the PLWS locus at 15q12. Our examination of a small series of AS patients without visible 15q12 deletions may demonstrate the presence of submicroscopic deletions that help to delimit the AS locus. These preliminary investigations, when compared to others, may facilitate the rapid molecular characterization of the locus and permit diagnosis

independent of/ complementary to subjective clinical criteria. The role of heterodisomal inheritance in AS is also assessed.

PROPOSITION FIVE

The Relationship of Myoclonic Epilepsy with Ragged Red Fibers (MERRF) to Mitochondrial Complex I and IV Defects

Introduction

Mitochondrial myopathies and encephalomyopathies are an increasingly diagnosed disease family responsible for a wide variety of muscular dystrophies and nervous system disorders. Age at symptomatic onset can range from infancy to late adulthood. Physiological manifestations can range from a mild, easy fatigability to severe, progressive, degenerative failures of skeletal and cardiac muscle, central and peripheral nervous systems and visceral organs. Specific syndromes are often defined by gross symptomology, such as in MELAS (Mitochondrial Encephalopathy with Lactic Acidosis and Stroke-like episodes) and Kearne-Sayre Syndrome (KSS), a multisystem disorder with adolescent onset of a progressive retinopathy pigmentosa, external ophthalmoplegia and heart block (138). Diagnosis of mitochondrial disease is based upon symptomatic presentation, tissue biopsy morphology and ultrastructure of muscle and mitochondria and biochemical testing (140).

Some disorders have been associated with specific defects of mitochondrial pathways or complexes (138,142). However, often not all individuals diagnosed as having a specific phenotype disorder demonstrate the specific defect and there exists a wide phenotypic variation even among patients who have been verified to have a given defect (ex: Leber Hereditary Optic Neuroretinopathy (LHON)) (139). Thus accurate diagnosis is complicated by a wide phenotypic expression of genotype. This is important as these

disorders require extensive utilization of the health care system and as some of these patients would benefit from dietary or pharmacological therapy that can partially bypass their defect if they could be accurately identified (147).

Investigations into the biochemical and genetic origin of these diseases are complicated by the nature of mitochondria. Mitochondria are formed from the products of two genetic systems, the nuclear genome and the mitochondrial (mt) genome. The 16.5 Kb human mitochondrial genome encodes the mitochondrial 16S rRNA, 12S rRNA, 22 tRNAs and 13 polypeptides of complexes I, III, IV and V. The remaining mitochondrial components are nuclearly encoded. Tissue-specific isozymes of nuclear-encoded subunits have been demonstrated (144,145). Thus, a specific defect may only be expressed in certain tissues. It has also been suggested that specific mitochondrial genomic defects might only be expressed when matched to a second, specific nuclear mutation, further obfuscating analysis (143). Muscle biopsy is frequently used diagnostically with tissue evaluated for abnormalities of mitochondrial size, shape, distribution, fine structure and inclusion bodies (138,140). Numerical increases of mitochondria in type I and type II muscle fibers result in a characteristic staining pattern, "Ragged Red Fibers (RRF)," using trichrome stains and enzyme-histochemistry. However, similar, morphologically abnormal mitochondria are common to many of these disorders and hence are not pathognomonic of a specific defect (138,140). Further, inclusion bodies and RRF can be found in healthy individuals (138,140,146) and not all mitochondrial disorders display them (ex: absence of RRF in Leigh's Syndrome). Biochemical analysis of mitochondrial defects is not generally available and wide variation in results can occur even in experienced hands (135).

Mitochondrial biochemical defects can be broadly divided into five categories: 1) defects of transport (ex: carnitine deficiency), 2) defects of substrate utilization, 3) defects in the Krebs cycle (ex: fumarase deficiency), 4) defects in oxidative phosphorylation coupling (ex: Luft's Syndrome) and, 5) defects in the respiratory chain (142). Defects encoded by the mitochondrial genome are confined to respiratory complexes I, III, IV and V. As mitochondria are maternally inherited, diseases caused by defects in their genome may be expected to fulfill four criteria: 1) maternal inheritance, 2) manifested as defects of oxidative phosphorylation, 3) extent of deficiency may vary along the maternal lineage as heteroplasmy of maternal and wild type mtDNA partition and, 4) different tissues should be affected sequentially as respiratory capacity decreases – CNS first, followed by type I muscle fibers, heart, kidney and liver.

Recently, one such disease, Myoclonic Epilepsy with Ragged Red Fibers (MERRF), has been reported to fulfill these criteria. It has been reported to be caused by mitochondria genomic defects in complexes I and IV (135). However, this report suffers many of the difficulties faced by the diagnostician: clinical tests poorly correlated to, or non-specific for, mitochondrial disorders, extreme variation in biochemical controls, and a wide phenotypic variation of affected individuals. Further, protein and restriction endonuclease analyses of the mtDNA failed to reveal mt genomic changes. It was concluded, based upon this data, that several uncharacterized DNA point mutations were responsible for MERRF. It is my opinion, however, based upon a review of the data, that these conclusions were unsubstantiated and that there is insufficient evidence at this time to ascribe MERRF to mtDNA encoded complex I and IV defects. Further, I feel that new technologies now exist that can definitively and rapidly assess the role that mtDNA plays in MERRF, and indeed, in all mtDNA-encoded

mitochondrial diseases.

Polymerase Chain Reaction (PCR) utilizes a thermal stable (Taq I) DNA polymerase that allows the rapid amplification of even very small quantities of DNA. Further, it allows direct DNA sequencing to be performed even on crude, uncloned DNA preparations (141). It has also been employed for the rapid analysis of point mutations in several systems (136,137) . As the DNA sequence of the human mitochondrial genome, and its variations are known (143), it should prove straight forward to couple these PCR techniques for the direct analysis of mtDNA in mitochondrial disorders. Once these mutations are identified, characterization of the corresponding mutant proteins or RNAs and their effect on metabolism and interaction with nuclear products can begin.

Proposal

The mitochondrial DNA from peripheral leukocytes of individuals suspected of having mitochondrially-encoded disease states (esp. MERRF) will be directly sequenced using PCR technology to determine the mutation, if any. While the entire mitochondrial genome can be sequenced, particular attention will be paid to sequences encoding subunits I, II and III of cytochrome c oxidase (complex IV), the cytochrome bc₁-complex subunit (complex III), the two F₁F₀ATP synthetase complex subunits (complex V) and the seven subunits of the NADH dehydrogenase (complex I). Suspected point mutations that are ambiguous by sequence analysis will be verified using PCR techniques. Mutant proteins identified in this way can then be molecularly characterized.

Experimental Approach

Previous evaluation of mitochondrial diseases have relied on difficult

clinical, morphological and biochemical tests. Here, rapid, direct PCR oligonucleotide-directed DNA sequencing of mtDNA will be employed to evaluate the contribution of these mtDNA mutations to disease. Briefly, mtDNA from crude, peripheral leukocyte DNA preparations will be amplified and sequenced either sequentially or concurrently (141). For MERRF, oligonucleotide primers from complex I subunits, ND1, 2, 3, 4c, 4, 5, and 6 and complex IV subunits Cc I, II, III will be first employed to determine if mutations exist which affect subunit proteins. Further, the entire 16.5 Kb mtDNA can be sequenced with little further effort.

Heteroplasmy of point mutant sequences can be evaluated at the 10% level by using degenerate oligonucleotide probes hybridized to PCR amplified DNA at stringent temperatures due to the thermal instability of base-pair mismatches (136) (heteroplasmy of at least 20% is thought to be necessary before a disease phenotype is clinically suspected). These may be suspected by the consistent inability to read definitive sequence at a nucleotide. Heteroplasmy of deletion mutants should be readily apparent from sequence irregularities. Using this method the entire genome may be sequenced on as few as 20 gels and the need for tissue biopsy specimens (both costly and objectionable to the patient) is eliminated. Mutant sequences can be molecularly cloned for further study.

The recent ability to microinject isolated mitochondria into mammalian cell lines raises exciting possibilities for complementation studies, in vitro.

Discussion

Mitochondrial diseases are increasingly being recognized as significant contributors to health care costs. A molecular understanding of these diseases

may facilitate treatment. Here the effects of mutations of the mtDNA in mitochondrial disease is directly addressed, both in terms of incidence and the affected proteins (or RNA). Identification of these mutant proteins will facilitate our ability to ask questions concerning protein-protein interaction and characterize the role of mitochondria in energy metabolism in human, *in vivo*. Further, the ability to identify mtDNA encoded mutations enhances our ability to isolate nuclear-encoded mutations (by elimination). Thus, the elaboration of the contribution of both genomes to human mitochondrial function, *in vivo*, is approachable.

REFERENCES

1. Brown, D. and Dawid, I. *Science* **160**, 272-280 (1968).
2. Spradling, A.C. and Mahowald, A.P. *Proc. Natn. Acad. Sci. U.S.A.* **77** 1096-2002 (1980) .
3. Schimke, R.T. *Cell* **37** 705-713 (1984).
4. Pimentel, E. *Cancer Genet. Cytogenet.* **14** 347-368 (1985).
5. Stark, G.R., Wahl, G.M. *Ann. Rev. Biochem.* **53** 447-491 (1984).
6. Cowel, J.K. *Ann. Rev. Genet.* **16** 21-59 (1982).
7. Schimke, R.T. Gene Amplification R.T. Schimke, ed., Cold Spring Harbor: New York: Cold Spring Harbor Laboratory (1982).
8. Barker, P.E. *Cancer Genet. Cytogenet.* **5** 81-94 (1982).
9. Kopnin, B.P., Massino, J.S. and Gudkov, A.V. *Chromosoma* **92** 25-36 (1985).
10. Alt, F.W., Keillems, R.E. and Schimke, R.T. *J. Biol Chem* **251** 3063-3074 (1976).
11. Flintoff, W.F., Weber, M.K., Nagains, C.R., Essani, A.K., Robertson, D. and Salser, W. *Mol. Cell. Biol.* **2** 275-285 (1982).
12. Melera, P.W., Lewis, J.A., Biedler, J.L. and Hession, C. *J. Biol. Chem.* **255** 7024-7028 (1980).
13. Masters, J., Keeley, B., Gay, H. and Attardi, G. *Mol. Cell. Biol.* **2** 498-507 (1982).
14. Srimatkandada, S., Medina, W.D., Cashmore, A.R., Whyte, W., Engel, D., Moroson, B.A. , Franco, C.T. , Dube, S . K. and Bertino, J . R. *Biochemistry* **22** 5774-5781 (1983).

15. Cowan, K.H., Goldsmith, M.E., Levine, R.M., Aitken, S.C., Douglass, E., Clendeninn, N., Neinhuis, A.W. and Lippman, M.E. *J. Biol. Chem.* **257** 15079-15086 (1982).
16. Wahl, G.M., Padgett, R.A. and Stark, G.R. *J. Biol. Chem.* **254** 8679-8689 (1979).
17. Brennard, J., Chinault, A.C., Konecki, D.S., Melton, D.W. and Caskey, C.T. *Proc. Nat. Acad. Sci. U.S.A.* **79** 1950-1954 (1982).
18. Chin, D.J., Luskey, K.L., Anderson, R.G.W., Faust, J.R., Goldstein, J.L. and Brown, M.S. *Proc. Nat. Acad. Sci. U.S.A.* **79** 1185-1189 (1982).
19. Yeung, C-Y., Ingolia, D.E., Bobonis, C., Dunbar, B.S., Riser, M.E., Siciliano, M.J. and Kellems, R.E. *J. Biol. Chem.* **258** 8338-8345 (1983).
20. McConlogue, L., Gupta, M., Wu, L. and Coffino, P. *Proc. Nat. Acad. Sci. U.S.A.* **78** 4985-4989 (1984).
21. Beach, L.R. and Palmiter, R.D. *Proc. Nat. Acad. Sci. U.S.A.* **78** 2110-2114 (1981).
22. Stallings, R. , Munk, A.C., Longmire, J.L., Hildebrand, C. E. and Crawford, B.D. *Mol. Cell. Biol.* **4** 2932-2936 (1984).
23. Gill, G.N., Weber, W., Thompson, D.M., Lin, C., Evans, R.M., Rosenfeld, M.G., Gamou, S. and Shimizu, N. *Somat. Cell. Mol. Genet.* **11** 309-318 (1985) .
24. Baskin, F., Rosenberg, R.N., and Dev, V. *Proc. Nat. Acad. Sci. U.S.A.* **78** 3654-3658 (1981) .
25. Riordan, J.R., Deuchars, K., Kartner, N., Noa, A., Trent, J. and Ling, V. *Nature* **316** 817-819 (1985).
26. Van der Blik, A.M., Van der Velde-Koerts, T., Ling, V. and Borst, P. *Mol. Cell. Biol.* **6** 1671-1678 (1986).

27. Roninson, I.B., Chin, J.E., Choi, K., Gros, P., Housman, D.E., Fojo, A., Shen, D-W, Gottesman, M.M. and Pastan, I. *Proc. Nat. Acad. Sci. U.S.A.* **83** 4538-4542 (1986).
28. Wong, A.J., Ruppert, J.M., Eggleston, J., Hamilton, S.R., Baylin, S.B., Vogelstein, B. *Science* **233** 461-464 (1986).
29. Brodeur, G.M., Seeger, R.C., Scwab, M., Varmus, H.E. and Bishop, M. *Science* **224** 1121 - 1124 (1984) .
30. Shiloh, Y., Shipley, J., Brodeur, G.M., Bruns, G., Korf, B., Donlon, T., Schreck, R.R., Seeger, R., Sakai, K. and Latt, S.A. *Proc. Nat. Acad. Sci.* **82** 3761 -3765 (1985) .
31. Fujita, J., Srivastava, S.K., Kraus, M.H., Rhim, J.S., Tronick, S.R. and Aaronson, S.A. *Proc. Nat. Acad. Sci. U.S.A.* **82** 3849-3853 (1985).
32. King, C.R., Kraus, M.H. and Aaronson, S.A. *Science* **229** 974-976 (1985).
33. Curt, G.A., Carney, D.N., Cowan, K.H., Jolivet, J., Bailey, B.D., Drake, J.C., Kao-Shan, C.S., Minna, J.D. and Chabner, B.A. *N. Eng. J. Med.* **208** 199-202 (1983).
34. Carman, M.D., Schornagel, J.H., Rivest, R.S., Srimatkandada, S., Portlock, C.S., Duffy, T. and Bertino, J.R. *J. Clin. Oncol.* **2** 16-20 (1984).
35. Trent, J.M., Buick, R.N., Olson, S., Horns, D.C. and Schimke, R.T. *J. Clin. Oncol.* **2** 8-15 (1984).
36. Bell, D.R., Gerlach, J.H., Kartner, N., Buick, R.N. and Ling, V. *J. Clin. Oncol.* **3** 311-315 (1985).
37. Biedler, J.L., Melera, P.W. and Spengler, B.A. *Cancer Genet. Cytogenet.* **2** 47-60 (1980).

38. Nunberg, J.H., Kaufman, R.J., Schimke, R.T., Urlaub, G. and Chasin, L.A. Proc. Nat. Acad. Sci. U.S.A. **75** 5553-5556 (1978).
39. Looney, J.E. and Hamlin, J.L. Mol. Cell. Biol. **7** 569-577 (1987).
40. Bostock, C.J. and Clark, E.M. Cell **19** 709-715 (1980).
41. Debatisse, M., Hyrien, O., Petit-Koskas, E., Robert de Saint-Vincent, B. and Buttin, G. Mol. Cell. Biol. **6** 1776-1781 (1986).
42. Ford, M. and Fried, M. Cell **45** 425-430 (1986).
43. Federspiel, N.A., Beverley, S., Schilling, J. and Schimke, R.T. J. Biol. Chem. **259** 9127-9140 (1984) .
44. Barker, P.E. Cancer Genet. Cytogenet. **5** 81-94 (1982).
45. Mark, J. Hereditas **57** 1-22 (1966).
46. Barker, P.E. and Stubblefield, E. J. Cell. Biol. **83** 663-666 (1979).
47. Hamkalo, B., Farnham, P.J., Johnston, R. and Schimke, R.T. Proc. Nat. Acad. Sci. U.S.A. **82** 1126-1130 (1985).
48. Levan, A. and Levan, G. Hereditas **88** 81-92 (1978).
49. Levan, G., Mandahl, N., Bregula, U., Klein, G. and Levan, A. Hereditas **83** 83-90 (1976).
50. Barker, P.E., Drwinga, H.L., Hittelman, W.N. and Maddox, A.M. Exp. Cell. Res. **130** 353-360 (1980).
51. Smith, G.R. Science **191** 528-535 (1976).
52. Schimke, R.T., Kaufman, R.J., Alt, F.W. and Kellems, R.E. Science **202** 1051 -1055 (1978).
53. Botchan, M., Topp, W. and Sambrook, J. Cold Spring Harbor Symp. Quant. Biol. **43** 709-719 (1978).
54. Osheim, Y.N. and Miller, Jr., O.L. Cell **33** 543-553 (1983).

55. Passananti, C., Davies, B., Ford, M. and Fries, M. *EMBO* **6** 1691-1703 (1987).
56. Tartof, K.D. *Proc. Nat. Acad. Sci. U.S.A.* **71** 1272-1276 (1974).
57. Hourcade, D., Dressler, D. and Wolfson, J. *Cold Spring Harbor Symp. Quant. Biol.* **38** 537-550 (1974).
58. Orr-Weaver, T. and Spradling, A.C. *Mol. Cell. Biol.* **6** 4624-4633 (1986).
59. Spradling, A.C., de Cicco, D.V., Wakimoto, B.T., Levine, J.F., Kalfayan, L.J. and Cooley, L. *EMBO* **6** 1045-1053 (1987).
60. Roberts, J.M., Buck, L.A. and Axel, R. *Cell* **33** 53-63 (1983).
61. Mariani, B.D. and Schimke, R.T. *J. Biol. Chem.* **259** 1901-1910 (1984).
62. Morgan, W.F., Bodycote, J., Fero, M.L., Hahn, M.L., Kappl, N., Pantelias, G.E. and Painter, R.B. *Chromosoma* **93** 191-196 (1986).
63. Brown, P.C., Tristy, T.D. and Schimke, R.T. *Mol. Cell. Biol.* **3** 1097-1107 (1983).
64. Varshavsky, A. *Cell* **25** 561-572 (1981) .
65. Tisty, T.D., Brown, P.C. and Schimke, R.T. *Mol. Cell. Biol.* **4** 1050-6 (1984).
66. Barsoum, J. and Varshavsky, A. *Proc. Nat. Acad. Sci. U.S.A.* **80** 5330-5334 (1983).
67. Smith, C.A. and Vinograd, J. *J. Mol. Biol.* **69** 163-178 (1972).
68. Henderson, G.B., Suresh, M.R., Vitols, K. and Huennekens, F. *Cancer Research* **46** 1639-1643 (1986).
69. Wolman, S.R., Craven, M.L., Grill, S.P., Domn, B.A. and Cheng, Y-C. *Proc. Nat. Acad. Sci. U.S.A.* **80** 807-809 (1983).
70. Gross-Bellard, M., Oudet, P. and Chambon, P. *Eur. J. Biochem.* **36** 32-38 (1973).

71. Morandi, C., Masters, J.N., Mottes, M. and Attardi, G. *J. Mol. Biol.* **156** 583-607 (1982).
72. Masters, J.N., Yang, J.K., Cellini, A. and Attardi, G. *J. Mol. Biol.* **167** 23-36 (1983).
73. Anagnou, N.P., O'Brien, S.J., Shimada, T., Nash, W.G., Chen, M-J. and Nienhaus, A.W. *Proc. Nat. Acad. Sci. U.S.A.* **81** 5170-5174 (1984).
74. Harper, M.E. and Saunders, G.F. *Chromosoma* **83** 431-439 (1981) .
75. Human Gene Mapping 8, *Cytogenet. Cell Genet.* **40** 492-535 (1985).
76. Carroll, S.M., Gaudray, P., De Rose, M.L., Emery, J.F., Meinkoth, J.L., Nakkim, E., Subler, M., Von Hoff, D.D. and Wahl, G.M. *Mol. Cell Biol.* **7** 1740-1750 (1987).
77. Bostock, C.J. and Tyler-Smith, C. *J. Mol. Biol.* **153** 219-236 (1981).
78. Nagl, W. *Endoploidy and Polyteny in Differentiation and Evolution* North-Holland, Amsterdam (1978).
79. Schwarzacher, H.G. and Schendl, W. *Cytogenetics* **4** 1-18 (1965).
80. Schmid, W. *Expl. Cell. Res.* **42** 201-204 (1966).
81. Levan, A. and Hsu, T.C. *Hereditas* **47** 69-71 (1961).
82. Ising, V. and Levan, A. *Acta. Pathol. Microbiol. Scand.* **40** 13-24 (1957).
83. Levan, A. and Hauschka, T.S. *J. Nat. Cancer Inst.* **14** 1-43 (1953).
84. Sarto, G.E., Stubblefield, P.A. and Therman, E. *Hum. Genet.* **62** 228-232 (1982).
85. Bamezai, R. and Shiraish, Y. *Hum. Genet.* **75** 239-243 (1987).
86. Schizuyo, S. and Tokuyama, F. *Cancer Res.* **34** 2615-2623 (1974).
87. Takanari, H., Nakakuki, K. and Izutsu, E. *Cytogenet. Cell Genet.* **39** 93-98 (1985).

88. Sperling, K. and Rao, P.N. *Chromosoma* **45** 121-131 (1974).
89. Sperling, K. and Hameister, H. *Chromosoma* **90** 389-393 (1984).
90. Rao, P.N. and Johnson, R.T. *Methods in Cell Physiology* **5** 75-126 (1972) .
91. Balaban-Malenbaum, G. and Golbert, F. *Cancer Genet. Cytogenet.* **2** 339-348 (1981) .
92. Hyrien, O., Debatisse, M., Buttin, G. and Robert de Saint Vincent, B. *EMBO J.* **6** 2401-2408 (1987).
93. Hyrien, O., Debatisse, M., Buttin, G. and Robert de Saint Vincent, B. *EMBO J.* **7** 407-417 (1988) .
94. Yamagishi, H., Kunisada, T. and Tsuda, T. *Plasmid* **8** 299-306 (1982).
95. Flores, S.C. , Sunnerhagen, P., Moore, T.K. and Gaubatz, J .W. *Nucl . Acids Res.* **16** 3889-3906 (1988).
96. George, D.L., and Powers, V.E. *Proc. Natl. Acad. Sci. U.S.A.* **79** 1597-1601 (1982).
97. Pauletti, G., Lai, E. and Attardi, G. *Proc. Natl. Acad. Sci. U.S.A.* **87** 2955-2959 (1990).
98. Wahl, G. *Cancer Res.* **49** 1333-1340 (1989).
99. Stark, G.R., Debatisse, M., Giulotto, E. and Wahl, G.M. *Cell* **57** 901-908 (1989).
100. Saito, I., Groves, R., Giulotto, E., Rolfe, M. and Stark, G.R. *Mol. Cell. Biol.* **9** 2445-2452 (1989).
101. Giulotto, E., Saito, I. and Stark, G.R. *EMBO J.* **5** 2115-2121 (1986).
102. Ford, M. and Fried, M. *Cell* **45** 425-430 (1986).
103. Ruiz, J. and Wahl, G.M. *Mol. Cell. Biol.* **8** 4302-4313 (1988).

104. Rouah, E., Wilson, D., Armstrong, D. and Darlington, G. *Cancer Res.* **49** 1797-1801 (1989).
105. Fukumoto, M., Estensen, R., Sha, L., Oakley, G., Twiggs, L. and Adcock, L. *Cancer Res.* **49** 1693-1697 (1989) .
106. Carroll, S., DeRose, M., Gaudray, P., Moore, C., Needham-Vandevanter, D., Von Hoff, D. and Wahl, G. *Mol. Cell. Biol.* **8** 1525-1533 (1988).
107. Ruiz, J., Choi, K., Von Hoff, D., Roninson, I. and Wahl, G. *Mol. Cell. Biol.* **9** 109-115(1989).
108. Hunt, J., Valentine, M. and Tereba, A. *Mol. Cell. Biol.* **10** 823-829 (1990)
109. Lapidot-Lifson, Y., Prody, C., Ginzberg, D., Meytes, H., Zakut, H. and Soreq, H. *Proc. Natl. Acad. Sci. U.S.A.* **86** 4715-4719 (1989).
110. Prody, C., Dreyfus, P., Zamir, R., Zakut, H. and Soreq, H. *Proc. Natl. Acad. Sci. U.S.A.* **86** 690-694 (1989).
111. Chandar, N., Lombardi, B. and Locker, J. *Proc. Natl. Acad. Sci. U.S.A.* **86** 2703-2707 (1989).
112. Shima, H., Nakayasu, M., Aonuma, S., Sugimura, T. and Nagao, M. *Proc. Natl. Acad. Sci.* **86** 7442-7445 (1989).
113. Nakayasu, M., Shima, H., Aonuma, S., Nakagama, H., Nagao, M. and Sugimura, T. *Proc. Natl. Acad. Sci.* **85** 9066-9070 (1988).
114. Tisty, T. *Proc. Natl. Acad. Sci.* **87** 3132-3136 (1990).
115. Wright, J., Smith, H., Watt, F., Hancock, C., Hudson, D. and Stark, G. *Proc. Natl. Acad. Sci.* **87** 1791-1795 (1990).
116. Von Hoff, D., Needham-VanDevanter, D., Yucel, J., Windle, B. and Wahl, G.M. *Proc. Natl. Acad. Sci.* **85** 4804-4808.
117. Prader-Willi Conference Report *Am. J. Med. Genet.* **28** 779-915 (1987).

118. Lubinsky, M., Zellweger, H., Greenswag, L., Larson, G., Hansmann, I. and Ledbetter, D. *Am. J. Med. Genet.* **28** 37-43 (1987).
119. Butler, M. *Am. J. Hum. Genet.* **45** 140-146 (1989).
120. Butler, M., Meaney, F. and Palmer, C. *Am J. Med. Genet.* **23** 793-809 (1986) .
121. Nichols, R., Knoll, J., Butler, M., Karam, S. and Lalande, M. *Nature* **34** 281-285 (1989).
122. Umadevi, T., Nicholls, R., Stroh, H., Ringer, S., Neve, R., Kaplan, L., Wharton, R., Wurster-Hill, D., Graham, J., Cantu, E., Frias, J., Kouseff, B. and Latt, S. *Am J. Med. Genet.* **33** 78-87 (1989).
123. Buiting, K., Neumann, M., Ludecke, H., Senger, G., Claussen, U., Antich., J., Passarge, E. and Horsthemke, B. *Genomics* **6** 521-527 (1990).
124. Williams, C., Gray, B., Hendrickson, J., Stone, J. and Cantu, E. *Am. J. Med. Genet.* **32** 339-345 (1989).
125. Pemprey, M., Fennell, S., Van Den Berghe, J., Fitchett, M., Summers, D., Butler, L., Clarke, C., Griffiths, M., Thompson, E., Super, M. and Baraister, M. *J. Med. Genet.* **26** 73-77 (1989).
126. Fisher, J., Burn, J., Alexander, F. and Gardner-Medwin, D. *J. Med. Genet.* **24** 294-298 (1987).
127. Boyd, S., Harden, A. and Patton, M. *Eur. J. Pediatr.* **147** 508-513 (1988).
128. Bjerre, I., Fagher, B., Ryding, E. and Rosen, I. *Acta. Paediatr. Scand.* **73** 398-402 (1984).
129. Williams, C., Hendrickson, J., Cantu, E. and Donlon, T. *Am. J. Med. Genet.* **32** 333-338 (1989).

130. Cooke, A., Tolmie, J., Glencross, F., Boyd, E., Clarke, M., Day, R., Stephenson, J. and Conner, J. *Am. J. Med. Genet.* **32** 545-549 (1989).
131. Nicholls, R., Knoll, J., Glatt, K., Hersh, J., Brewster, T., Graham, J., Wurster-Hill, D., Wharton, R. and Latt, S. *Am J. Med. Genet.* **33** 66-77 (1989).
132. Robb, S., Pohl, K., Baraitser, M., Wilson, J. and Brett, E. *Arch. Dis. Child.* **64** 83-86 (1989).
133. Willems, P., Dijkstra, I., Brouwer, O. and Smit, G. *Am. J. Med. Genet.* **27** 773-780 (1987).
134. Human Gene Mapping 10 Report *Cytogenet. Cell. Genet.* **51** 282, 777 (1989).
135. Wallace, D., Zheng, X., Lott, M., Schoffner, J., Hodge, J., Kelley, R., Epstein, C. and Hopkins, L. *Cell* **55** 601-610 (1988).
136. Farr, C., Saiki, R., Erlich, H., McCormick, F. and Marshall, C. *Proc. Natl. Acad. Sci.* **85** 1629-1633 (1988).
137. Saiki, R., Bugawan, T., Horn, G., Mullis, K. and Erlich, H. *Nature* **324** 163-166 (1986).
138. Goebel, H., Bornemann, A. and Reichmann, H. *Neuropath. Appl. Neurobio.* **15** 97-119 (1989).
139. Vikki, J., Savontraus, M-L. and Nikoskelain, E. *Am. J. Hum. Genet.* **45** 206-211 (1989).
140. Stadhouder, A. and Sengers, R. *J. Inher. Metab. Dis.* **10** Suppl.1 62-80 (1987).

141. Brow, M. In PCR Protocols M. Innis, et al., ed. Academic Press San Diego 189-196 (1990).
142. DiMauro, S., Bonilla, E., Zeviani, M., Servidei, S., DeVivo, D. and Schon, E. J. Inher. Metab. Dis. **10** Suppl. 1 113-128 (1987).
143. Tager, J. J. Inher. Metab. Dis. **10** Suppl. 1 3-10 (1987).
144. Sinjorgo, K., Hakvoort, T., Muijsers, A., Schram, A. and Tager, J. J. Inher. Metab. Dis. **11** Suppl. 2 202-204 (1988).
145. Kroon, A. and Van den Bogert, C. J. Inher. Metab. Dis. **10** Suppl. 1 54-61 (1987).
146. Sengers, R. and Stadhouders, A. J. Inher. Metab. Dis. **10** Suppl. 1 98-104 (1987).
147. Przyrembel, H. J. Inher. Metab. Dis. **10** Suppl. 1 129-146 (1987).

Publications

Papers

- 1) Maurer, B.J., Lai, E., Hamkalo, B., Hood, L.E. and Attardi, G.: Novel Submicroscopic Element Containing Amplified Genes in Human Cells. *Nature* **327** 434-437 (1987).
- 2) Maurer, B.J., Carlock, L., Wasmuth, J.J. and Attardi, G.: Assignment of Human Dihydrofolate Reductase Gene to Band q23 of Chromosome 5 and of a Related Pseudogene Psi-HD1 to Chromosome 3. *Somat. Cell. Mol. Genet.* **11** 79-85 (1985).
- 3) Maurer, B.J., Barker, P.E., Masters, J.N., Ruddle, F.H. and Attardi, G.: Human Dihydrofolate Reductase Gene is Located in Chromosome 5 and is Unlinked to the Related Pseudogenes. *Proc. Natl. Acad. Sci. U.S.A.* **81** 1484-1488 (1984).

Abstracts

- 1) Lai, B., Maurer, B., Hamkalo, B., Hood, L. and Attardi, G.: Identification of Novel Submicroscopic Extrachromosomal Elements Containing Amplified Genes in Human Cells by Pulsed-Field and Field-Inversion Gel Electrophoresis. *Leukemia* **1** 276 (1987).
- 2) Maurer, B., Barker, P.E., Masters, J.N., D'Eustachio, P., Ruddle, F.H. and Attardi, G.: Chromosomal Location of the Normal Human DHFR Gene and of its Amplified Copies in Methotrexate Resistant Cell Variants. *HMG 7 Cytogenet. Cell. Genet.* **37** 534 (1984).