A Study of Chromosomal Changes Associated with Amplified Dihydrofolate Reductase Genes in Rat Hepatoma Cells and Their Dedifferentiated Variants

CATHERINE FOUGERE-DESCHATRETTE,*‡ ROBERT T. SCHIMKE,* DOMINIQUE WEIL,§ and MARY C. WEISS‡

*Department of Biological Sciences, Stanford University, Stanford, California 94305; ‡Centre de Génétique Moléculaire du Centre National de la Recherche Scientifique, 91190 Gif-sur-Yvette, France; §Unité de Recherche de Génétique Médicale du Institut National de la Science Expérimentale et de la Recherche Médicale, U12, Hôpital des Enfants Malades, 75730 Paris Cédex 15, France

ABSTRACT We have examined the karyological consequences of dihydrofolate reductase gene amplification in a series of six rat hepatoma cell lines, all derived from the same clone. Cells of three of these lines express a series of liver-specific functions whereas those of three others fail to express these functions. Cells of each line have been subjected to stepwise selection for methotrexate resistance and, in most cases, resistance is associated with a 40–50-fold amplification of sequences hybridizing to a dihydrofolate reductase cDNA probe. In one line no modified chromosome is observed, whereas in two others the amplified genes are associated with an expanded chromosomal region. R-banding analysis of these karyotypes showed that few changes have occurred. These observations apply to two of the well-differentiated lines, and to a variant able to revert to the differentiated state. In contrast, in the two stably dedifferentiated hepatoma cell lines, amplified dihydrofolate reductase genes are found on large chromosomes of variable size, on ring chromosomes, and on chromosomes containing terminal, median, or multiple centromeres. We conclude that the nature of the chromosomal changes associated with dihydrofolate reductase gene amplification are the result of differences in cell lines rather than in the protocols employed for selection.

Amplified dihydrofolate reductase (DHFR)[1] genes in mouse, hamster, and human cells are associated with a wide variety of karyotypic alterations, including their presence on one or more chromosomes as an expanded region (2, 3, 12, 26, 27), their presence in extensively rearranged chromosomes (22, 32), or as extrachromosomal elements or double minute chromosomes (4, 21). The various configurations of amplified sequences may be due to differences in mechanisms underlying the amplification process, differences in selection procedures employed in various laboratories, or to the use of different cell lines.

To determine whether the nature of chromosomal aberrations associated with DHFR gene amplification is a function of cell type or of selection protocol, we have studied a set of sister clones with different phenotypes derived from a clonal, rat hepatoma cell line. Within this relatively constant genetic and variable epigenetic background, we have compared the chromosomal changes that occur during the acquisition of resistance to methotrexate (MTX) using a single selection protocol. The cells used include three clones of well-differentiated cells characterized by the expression of a number of proteins specific to hepatocyte differentiation and three variant clones that are deficient in the expression of hepatocyte functions (10). We do find a pattern of differences in the structure of chromosomes with amplified DHFR genes in the various cell lines, and suggest that the nature of the chromosomal aberrations obtained is a function of the phenotype of the cells.

[1] *Abbreviations used in this paper:* DHFR, dihydrofolate reductase; MTX, methotrexate.

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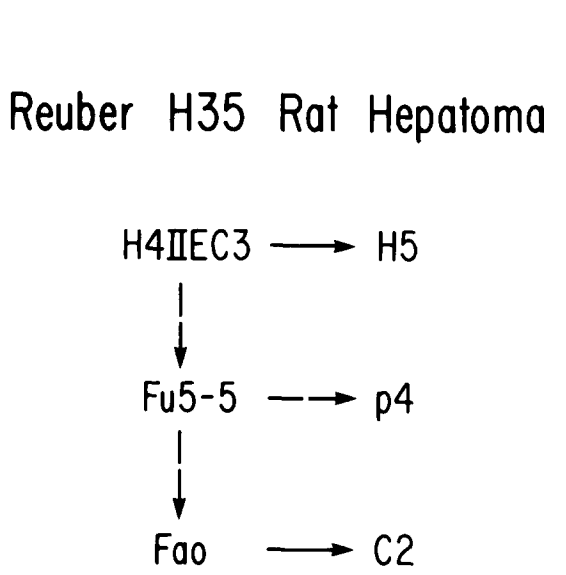

FIGURE 1 Genealogy of the rat hepatoma clones and their variants. On the left are the well-differentiated clones, and on the right are their variants. The unbroken arrows indicate that the clone is a direct descendant of the immediate progenitor on the figure, and the broken arrow signifies that intermediate clones (not shown) exist. H5 cells arose during an attempt to select H4IIEC3 cells that can grow in suspension: after a number of passages the culture became heterogeneous, and subcloning led to the isolation of H5. p4 cells appeared in a twice-cloned population of Fu5-5 cells that had been selected for resistance to eight azaguanine. C2 cells were observed as a morphologic variant and immediately cloned from a freshly thawed culture of Fao cells.

MATERIALS AND METHODS

Cell Lines and Culture Conditions: The six hepatoma clones are all derivatives of line H4IIEC3 of Pitot et al. (28), adapted to growth in vitro from the Reuber H35 hepatoma (29). Their derivation is shown in Fig. 1. Clones H4II, Fu5-5, and Fao express functions characteristic of hepatic differentiation, whereas H5, p4, and C2 cells fail to do so (10). Reversion to the differentiated state has been obtained for derivatives of C2, but not of H5 and p4 (8, 9 and unpublished results). Cells were grown in modified (7) Ham's F12 medium (20) lacking hypoxanthine and thymidine, supplemented with 5% fetal calf serum (GIBCO Laboratories, Santa Clara, CA). Although the various clones used here were isolated at widely different times (10, 28), the cells have not been in continuous culture. Immediately following the isolation of each clone, a large number of ampules was frozen. All experiments were undertaken with freshly unfrozen cells.

Methotrexate Selection: The same protocol was used for all six clones. First a series of petri dishes was seeded with $1-2.5 \times 10^4$ cells/cm^2 with concentrations of MTX of 1–100 nM. This range of concentrations bracketed responses varying from no effect to complete killing of cells. Dishes with some surviving cells (in most cases at 20 nM MTX) were allowed to recover by one or two passages at this MTX concentration, and further selection was pursued by plating $1-4 \times 10^4$ cells/cm^2 in two- (or four-) fold increments in MTX concentration. This stepwise process, including two passages of cells at the given MTX concentration, was continued and cells were finally obtained with resistance to 2–20 μM MTX. During the course of this stepwise selection, MTX resistant populations were frozen at −80° to permit analysis of the progression of DHFR enzyme content, DHFR gene copy number, and karyotypic changes.

Stability of MTX Resistance: $1-2 \times 10^5$ MTX-resistant cells were plated into MTX-free medium, and passaged every week at a 1:100 dilution over 60 cell generations. At 20, 40, and 60 cell generations, the DHFR enzyme content was determined. At 60 cell doublings in the absence of MTX, 2×10^5 cells were seeded in medium containing MTX at the concentration to which cells were resistant when the stability test was started.

Determination of DHFR Enzyme Content: DHFR enzyme content was determined using the stoichiometric binding of [^{3}H]MTX to DHFR (18). Free [^{3}H]MTX was removed from the bound fraction by the addition of activated charcoal coated with dextran and bovine serum albumin. Resistant cells were grown for 72 h in MTX-free medium before assay to free the DHFR from binding to MTX.

Estimation of DHFR Gene Amplification: The blot hybridization technique of Gasser et al. (17) was employed. This method allows for a measure of differential DHFR gene amplification; genomic DNA samples are hybridized to both a ^{32}P-labeled DHFR cDNA clone (6) and to a serum albumin ^{32}P-labeled cDNA (supplied by J. Taylor, Gladstone Research Institute, San Francisco, CA). The hybridization intensities are compared to densitometric scanning in a linear response range (see Brown et al. [5]).

Karyotype Analyses: Karyotypes were performed on colchicine or colecimide-arrested metaphases by the standard air-drying technique and were treated for R-binding (13). Localization of DHFR genes was determined by in situ hybridization of iodinated DHFR cDNA as described previously (21).

RESULTS

Properties of Methotrexate Resistant Hepatoma Cell Lines

Fig. 1 provides the geneology of the six hepatoma clones. H4II is the original Reuber H35 clone of Pitot et al. (28) from which was derived successively Fu5-5 and Fao. H4II gave rise to the dedifferentiated variant H5, whereas p4 cells derive from Fu5-5. Dedifferentiated variant Faofl C2 (referred to as C2), a descendant of Fao cells, reverts to the differentiated state at a spontaneous frequency of 10^{-8} and can be induced to undergo partial reversion at a frequency of $1-6 \times 10^{-2}$. Thus far it has not been possible to obtain revertants of H5 and P4 (see Deschatrette et al. [9] for details).

Cells of all six clones were subjected to the same protocol of stepwise selection for MTX resistance using twofold or fourfold increments in drug concentration. Table I gives a summary of the properties of the sensitive lines and the MTX-resistant populations derived from them. In most cases resistance to 1–2 μM MTX is associated with a 40–50-fold amplification of the DHFR gene, and a 100–200-fold increase in the amount of DHFR enzyme activity. Of the six cell lines only one, H4II, gave rise to resistant cells in the absence of significant DHFR gene amplification. Similarly, Galivan (16) selected MTX-resistant Reuber H35 cells (the same cell line we denote as H4II), and obtained cells with modified MTX

TABLE I
Properties of Sensitive and MTX-resistant Cells

Cell line	DHFR/mg of protein*	Fold-amplification of DHFR genes‡	No. of chromosomes§
	μmol/min/mg		
H4II	22	1	50 (47–57)
H4II 2 μM MTX	93 (4 ×)	3‖	52 (48–56)
Fu5-5	18	1	52 (50–53)
Fu5-5 1 μM MTX	2,400 (1,330)	45	51 (48–54)
Fao	20	1	52 (50–55)
Fao 1 μM MTX	2,160 (110 ×)	41	50 (46–51)
H5	20	1	53 (50–56)
H5 2 μM MTX	3,400 (160 ×)	40	51 (45–53)
p4	28	1	46 (39–49)
p4 20 μM MTX	2,080 (70 ×)	42	46 (39–49)
C2	8	1	51 (49–54)
C2 0.2 μM MTX	ND	10	52 (50–60)
C2 20 μM MTX	1,140 (140 ×)	56	52 (50–53)

* Specific activity: micromoles DHFR/minutes × 10^{-10}/milligram of soluble protein.
‡ Fold amplification relative to parental cell line.
§ Mode and range in parentheses; based upon the analysis of 15–30 metaphases.
‖ The method of detection of amplification level is sufficiently sensitive to detect an approximately threefold degree of amplification. A threefold amplification of the DHFR gene cannot be detected by in situ analysis of metaphase chromosomes employing the method used in this paper.

transport and little or no increase in DHFR enzyme activity. When we selected the H4II cells for resistance to higher MTX concentrations (120 μM), a 20–30-fold amplification of the DHFR gene was obtained, suggesting that mixed modes of MTX resistance (transport and DHFR gene amplification) can occur in the same cells. It is surprising and remains unexplained that only cells of line H4II, the progenitor clone, fail to undergo DHFR gene amplification at low to intermediate MTX concentrations.

The time required to obtain cell populations resistant to 2 μM MTX from the different cell lines differed by a factor of two (3–7 mo) when the MTX increments were twofold at each selection step. However, we found in subsequent experiments that when the increments were fourfold or greater, an obvious difference between cell lines was noted: only dedifferentiated H5 and p4 cells gave resistant progeny readily. Thus, with some cell lines, attempts to obtain resistance and gene amplification may fail when the step increments are large.

Localization of Amplified Dihydrofolate Reductase Genes

Karyotype analyses and in situ hybridization to localize the amplified DHFR genes have been undertaken on each of the resistant populations. We present here results concerning intermediate levels of resistance (0.2–40 μM) with the aim of observing chromosomal changes in the absence of secondary mutations (superimposition of transport [16, 30] or DHFR affinity [14, 18]) that may occur in addition to initial gene amplification events. In no cases were double minute chromosomes observed, and growth of the resistant cells in the absence of MTX for 60 generations did not result in a significant reduction in amplified DHFR genes or DHFR enzyme content (data not shown).

Fig. 2 shows representative metaphase spreads after hybridization to a iodinated mouse DHFR cDNA probe (6). Additional metaphase spreads showing in situ localization of amplified DHFR genes in these cell lines have been published in a preliminary report (15). Three classes of labeled chromosomes were observed. (*a*) In Fao cells the majority of cells (60%) contained a single chromosome carrying DHFR genes. In spreads where the labeling did not obscure it, this small chromosome appeared as a metacentric which differed in no obvious way from the small metacentric chromosomes of sensitive Fao cells (see Fig. 3). (*b*) In Fu5-5 and C2 cells, DHFR genes were localized to one (90%) or two (10%) chromosomes that, in comparison with the karyotype of sensitive Fu5-5 and C2 cells, constituted a new, large, subtelocentric chromosome, whose length increased with higher levels of resistance (Fig. 2*b*, *c*). 3) Strikingly different results were obtained for H5 and p4 cells, the dedifferentiated, nonreverting variants. One to two chromosomes per cell (range one to three) containing DHFR genes were present, but they were highly variable from one metaphase to another in both size and structure, containing terminal, median, or multiple centromeres, or constituting ring structures. Examples of some of these structures are shown in Fig. 2*e–h*. The differences in DHFR containing chromosomes in the different populations were not paralleled by a significant difference in DHFR gene copy number (Table I).

Karyotypes of Sensitive and Methotrexate-resistant Cells

Metaphases of sensitive and resistant cells were subjected to thermal denaturation to obtain R-banding (13). Fig. 3 shows karyotypes from sensitive Fao cells, and the resistant

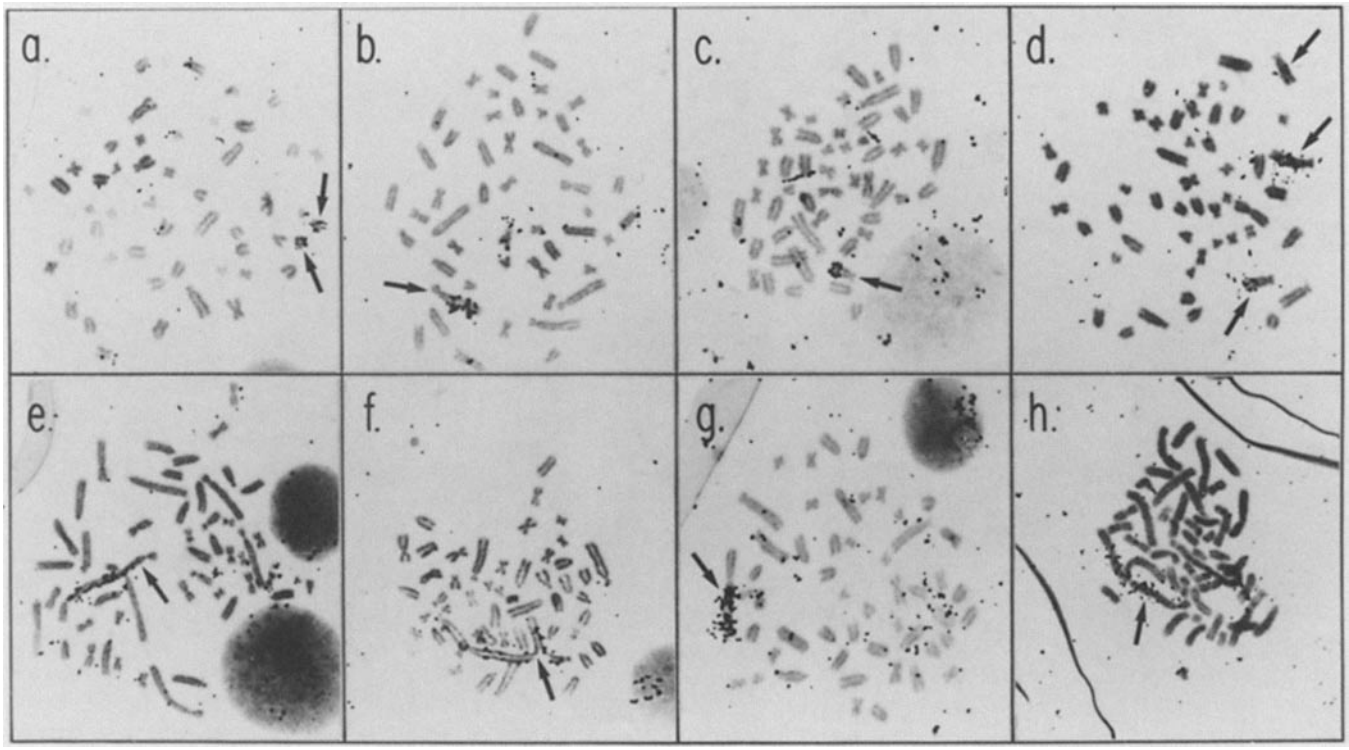

FIGURE 2 In situ hybridization of amplified DHFR genes. (*a*) Fao, resistant to 1 μM MTX; (*b*) Fu5-5, resistant to 1 μM MTX; (*c*) C2, resistant to 0.2 μM MTX; (*d*) C2 resistant to 20 μM MTX (note that the labeled chromosome is markedly longer in *d* than in *c*; (*e–g*) examples of metaphases of H5 cells resistant to 2 μM MTX; (*h*) p4, resistant to 40 μM MTX.

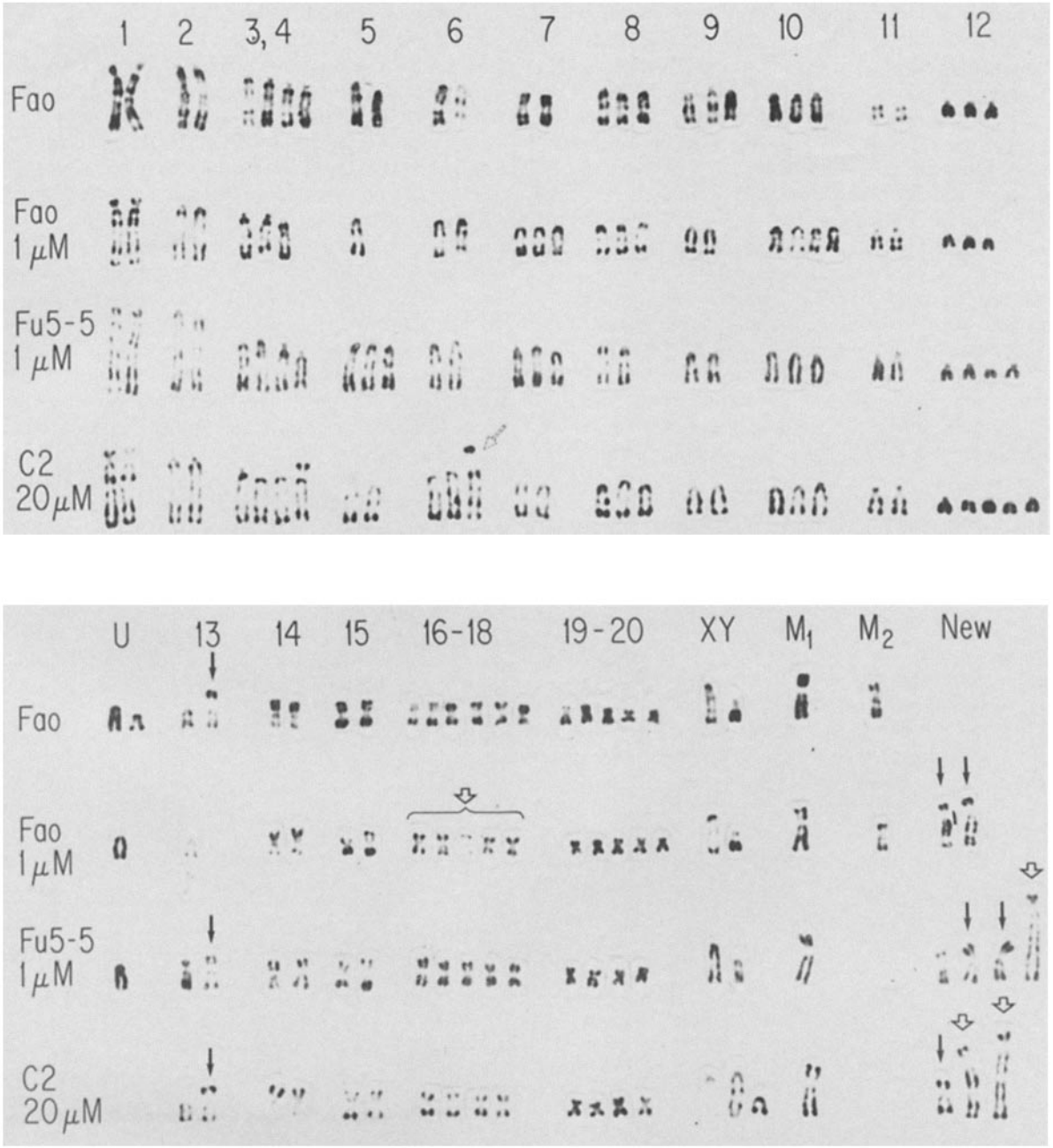

FIGURE 3 R-banded metaphases of Fao cells and of MTX-resistant cells of Fao, Fu5-5, and C2. Chromosomes are arranged according to Dev et al. (11). Some chromosomes are grouped, either when exact identification was not possible (*16–18* and *19–20*) or when translocations have occurred between neighbors (3, 4). *U* signifies unidentified chromosomes, M_1 and M_2 are frequently encountered marker chromosomes, and the category "New" includes markers found in resistant cells but not in their sensitive parent. Three types of arrows are used. Thin, black arrows denote the modified copy of chromosome 13 found in both sensitive and resistant cells, and on the right, the further modified chromosome 13 found in resistant cells. The dotted arrow shows the modified chromosome 6 containing a typical expanded region, but carrying no DHFR genes. The wide, white arrows show the chromosome(s) or chromosome group that carries amplified DHFR genes (see Fig. 2).

cells whose amplified genes are present on identifiable chromosomes, i.e., Fao, Fu5-5, and C2. Because the sensitive cells of Fao, Fu-5-5, and C2 show very similar karyotypes, we have chosen to present a metaphase from Fao, a widely used cell line, as representative. As can be seen in Fig. 3, in Fao most chromosomes are present in two or three copies; no systematic monosomies are observed. Contrasted to the karyotype of the Norway rat (11), from which the cell lines were ultimately derived, complex interchanges between chromosome 3 and 4 have occurred, giving rise to copies of chromosome 4 containing the short arms of chromosome 3, as well as other exchanges between the two chromosomes. A modified chromosome 13 (expanded short arms), an unidentified telocentric, and a large submetacentric chromosome (M1), whose long arms can be identified as chromosome 10 in G-banded metaphases, are regular features of these lines. In Fig. 3 the small metacentric chromosomes are grouped by size.

We consider now the karyotypes of resistant Fao, Fu5-5,

and C2 cells (Fig. 3). The karyotype of resistant Fao cells is very similar to that of the sensitive parent; one or two new subtelocentric chromosomes are observed (probably derived by the addition of material onto the already modified chromosome 13)—these chromosomes do not contain amplified DHFR genes. The amplified genes are present on chromosomes of the size and shape of pairs 16–18; no obvious modifications in size or banding patterns of these chromosomes can be detected. In Fu5-5 cells, where the amplified DHFR genes are present in a subtelocentric chromosome showing expanded, long arms, this new chromosome, lacking clearly defined bands, as well as newly modified copies of chromosome 13, can be seen; no other changes are evident. In C2 cells the DHFR-containing long, subtelocentric chromosome can be identified, as well as blocks of darkly staining material added onto chromosomes 4, 6, and most likely 13. The region between the long arms of chromosome 6 and its newly acquired satellites might be defined as homogeneously staining region (3); nevertheless, it does not contain amplified DHFR genes.

The new chromosome containing amplified DHFR genes in Fu5-5 and C2 cells has short arms compatible with its origin from one of the chromosomes of group 16–18, where all of the amplified genes are located in Fao cells. A reasonable interpretation of these observations is that the original DHFR gene is present in one of these chromosomes and that it is amplified in the absence of translocation in each of the three cell lines. This interpretation cannot be affirmed further, in that there is no simple method of identifying the chromosome(s) carrying the original DHFR genes in each of these clones. The modified chromosome 13 is further modified in resistant cells of the three independent lines; this is probably not a random change.

R-banded metaphases of resistant H5 cells (p4 was not studied) have proven difficult to interpret in the light of the results of in situ hybridization, and are not presented here. As shown in Fig. 2, DHFR containing chromosomes are very different from one metaphase to another. It is clear that multiple rearrangements accompany DHFR gene amplification in H5 and p4, and only in cells of these two variant lines. In MTX-resistant populations of both H5 and p4, a stem cell failed to emerge.

Expression of Differentiation by Sensitive and MTX-resistant Cells

An initial purpose of this work was to investigate the influence of DHFR gene amplification on the expression of differentiation. This was assessed by three criteria (see Deschatrette et al. [9] for methods): the production of serum albumin, activity and inducibility of tyrosine aminotransferase, and the ability of cells to proliferate in glucose-free medium, where survival requires activity of liver-specific gluconeogenic enzymes (1). Sensitive and MTX-resistant cells at various levels of resistance have been studied. Detailed results will not be presented inasmuch as no qualitative changes were observed. H4II, Fu5-5, and Fao cells, both sensitive and resistant, remained clearly positive by all three criteria. In addition, at all stages of selection they retained the morphological properties characteristic of well-differentiated hepatoma cells. Among the three variant lines only C2 cells were studied; by all criteria the resistant cells resembled the sensitive parental cells.

DISCUSSION

Our results show that the same protocol of MTX selection results in markedly different chromosome changes in closely related cell lines with similar numbers of DHFR genes, indicating that the karyological consequences of gene amplification are a function of cell type. Several points are worthy of comment. (*a*) In resistant Fao, Fu5-5, and C2 cells a single chromosome, occasionally present in two copies in a cell, contains the amplified DHFR genes. These DHFR-containing chromosomes appear to have all been generated from the same chromosome, i.e., a small metacentric chromosome, and they show variable lengths of the long (DHFR-containing) arm. In Fao cells no expansion of the DHFR-containing chromosome is evident. The degree of expansion of a chromosome is a function of both the gene copy number and the unit length of amplified DNA sequence, which can vary in independent cell isolates (26, 27). Thus the lack of expansion of a chromosome does not necessarily exclude the possibility of extensive gene amplification, in this case at least 40 copies of a gene. (*b*) In resistant C2 cells, but not in the sensitive parental cells, a modified chromosome 6 contains a nonbanding, expanded region, which does not contain DHFR genes (see Fig. 3). Thus the presence of an expanded region on a chromosome does not necessarily localize a potentially amplified gene for which selection has been accomplished. (*c*) In dedifferentiated H5 and p4 cells, multiple aberrations in DHFR-containing chromosomes have occurred, which is in contrast to the uniform chromosomal changes in Fao, Fu5-5, and C2 cells.

It is not clear why various cell lines derived from the same cell lineage generate different chromosomal aberrations under the same selection protocol and with the same number of amplified DHFR genes. In all of the hepatoma cell lines employed here, the karyotype is relatively stable, and in all cases the amplified genes are present on chromosomes. A stable karyotype and chromosomal localization of amplified DHFR genes is a phenomenon observed in many cell lines (3, 12, 26, 27), as well as with other amplified genes (25, 33). In contrast, cells derived from mouse fibroblasts which characteristically are highly aneuploid contain amplified DHFR genes as self-replicating, extrachromosomal elements, i.e., double minute chromosomes (4, 19, 21). The dedifferentiated variant hepatoma H5 and p4 cell lines appear to be intermediate in terms of chromosomal aberrations associated with DHFR gene amplification. Thus, although the DHFR genes are chromosomal, the DHFR-containing chromosomes are highly aberrant and take a variety of forms. Recent studies (24) show that when cells are subjected to treatments (such as hydroxyurea) that inhibit DNA synthesis, cells respond by overreplication (disproportionate replication) of a portion of the genome, including DHFR genes, in the same cell cycle. Upon subsequent MTX selection the DHFR genes are selectively retained in the genome. Thus in order for cells to survive, a number of recombination–repair events must take place to maintain a viable genome, some of which may result in various forms of chromosomal rearrangements. In this context it is intriguing to note that Loquet and Weibel (23) have found that H5 cells, contrasted to a Fao–H5 hybrid cell line, which retains liver-specific functions, are deficient in the ability to excise DNA damage introduced by alkylating agents. Whether this repair defect in the H5 cells is related to the extensive chromosomal aberrations in this cell line awaits

further study. An alternative explanation for the finding that only the differentiated and the reversion-competent variant cell lines have minimal chromosomal aberrations associated with DHFR gene amplification is that cells in the differentiated state cannot tolerate extensive chromosomal rearrangements and/or a high degree of alterations in gene dosage of DNA sequences co-amplified with DHFR genes.

These Reuber H35-derived cell lines show a number of properties that are associated with retention of the differentiated state and change with its loss. The differentiation state extends not only to the synthesis of hepatocyte-specific proteins, but also to cell morphology, and even to chromatin repeat length (31). The results reported here extend these observations to a new feature, the manner in which cells respond to environmental stress by remodeling their karyotypes. Thus, we have observed that differentiated hepatoma cells become resistant to MTX either by some change different from DHFR gene amplification (H4II), or they do undergo DHFR gene ampification, but with a minimum of karyotypic changes (Fao and Fu5-5). Only one variant dedifferentiated line shows the same pattern of karyotypic changes as the differentiated cells, and this is the only variant of the three tested that retains the capacity to revert to the differentiated state. From these four cases we observe a pattern: resistance to MTX is associated with little change in karyotype and with retention of the original phenotype. In contrast, only the variant lines H5 and p4 showed the kind of karyotypic reshuffling that we had anticipated might be incompatible with retention of the differentiated state.

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