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Genetics

Selective amplification of polymorphic dihydrofolate reductase gene loci in Chinese hamster lung cells

(gene amplification/genetic polymorphism)

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ABSTRACT A series of antifolate-resistant Chinese hamster lung sublines that overproduce either a M_r 20,000 or a M_r 21,000 class of dihydrofolate reductase (DHFR; tetrahydrofolate dehydrogenase; 5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3), known to contain amplified DHFR genes, has been analyzed by DNA and RNA transfer techniques. The results suggest that the M_r 20,000 and M_r 21,000 DHFRs are encoded by at least two polymorphic DHFR genes, both of which are expressed in drug-sensitive parental cells. In drug-resistant sublines only one of the two DHFR gene types is amplified, thus accounting for the overproduction of one or the other molecular weight class of DHFR. In addition to the known differences between the DHFRs whose overproduction they direct, these allelic genes differ in restriction endonuclease profiles and in the relative abundances of their multiple mRNA transcripts.

The amplification of dihydrofolate reductase (DHFR; tetrahydrofolate dehydrogenase; 5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase; EC 1.5.1.3) genes as a mechanism for antifolate resistance was postulated first on the basis of cytogenetic data by Biedler and Spengler (1) and subsequently was confirmed experimentally by Alt *et al.* (2) by using solution hybridization techniques. Gene amplification has been shown since to mediate the overproduction of DHFR as well as several other proteins in a wide variety of somatic cells maintained *in vitro* (3–10). In studying the DHFR overproduction phenomenon in the antifolate-resistant Chinese hamster lung cell system described by Biedler *et al.* (11), we observed that either a M_r 20,000 or a M_r 21,000 DHFR could be overproduced by different sublines that were independently derived from the same parental cell line, DC-3F (12). More recently (13), we have shown that of 16 drug-resistant sublines studied, 12 overproduced a M_r 21,000 DHFR and 4 overproduced a M_r 20,000 DHFR, whereas the drug-sensitive parental line synthesized control levels of both enzyme classes. To date, this report of DHFR molecular weight variance remains exceptional in an extensive literature of DHFR overproduction and gene amplification.

As a first step toward a genetic understanding of this DHFR variant phenomenon, we reported earlier the molecular cloning of a 700-base pair Chinese hamster DHFR double-stranded cDNA into pBR322 and the use of this recombinant plasmid as a hybridization probe in a RNA transfer analysis to demonstrate the presence of three poly(A)$^+$ DHFR mRNAs in antifolate-resistant Chinese hamster lung fibroblast lines—namely, 1,400, 2,200, and 3,300 bases (14). Moreover, we reported that in the DC-3F/MQ19 cell line that overproduced the M_r 20,000 DHFR, the smallest of these molecules—i.e., 1,400 bases—was the predominant DHFR mRNA, whereas the 3,300-base molecule was the predominant DHFR mRNA in the DC-3F/A3 cell line that overproduced the M_r 21,000 DHFR. Since that time we have analyzed several other sublines of the Chinese hamster lung series.

In this report we substantiate the correlation between the molecular weight of DHFR that is overproduced by drug-resistant cells and their DHFR mRNA size-distribution patterns. We demonstrate further that genomic DHFR DNA sequences from cells that overproduce M_r 20,000 DHFR can be distinguished by restriction analysis from the genomic DNA of cells that overproduce M_r 21,000 DHFR and, on the basis of these data, we conclude that the associated DHFR molecular weights and DHFR mRNA distribution profiles are phenotypic expressions of at least two polymorphic DHFR genes present in the DC-3F parental cell genome.

MATERIALS AND METHODS

Cell Lines and Culture. The parental cell line DC-3F was derived by Biedler and Riehm (15) by two sequential clonings of line Dede, which was established *in vitro* by T. C. Hsu from normal Chinese hamster lung tissue. DC-3F cells are near-diploid, substrate-attached, fibroblast-like cells. They are spontaneously transformed (16) and are sensitive to the antifolate drugs methotrexate and methasquin. All of the antifolate-resistant sublines used here were independently derived (i.e., from separate cultures of DC-3F) as described (11). Maintenance of cultures and harvesting of cells at midlogarithmic phase was as reported by Melera *et al.* (12).

Extraction and RNA Transfer Analysis of Polyadenylylated RNA. Cytoplasmic or polysomal polyadenylylated RNA was prepared as described by Melera *et al.* (12). Poly(A)$^+$ RNAs were denatured in formaldehyde/formamide, electrophoresed through 1.5% agarose gels (17), transferred by blotting either to diazobenzyloxymethyl-paper (18) or nitrocellulose paper (19), and probed with a ^{32}P-labeled, nick-translated, cloned Chinese hamster-specific DHFR double-stranded cDNA, pDHFR6 (14).

Preparation of High Molecular Weight DNA and Southern Blot Analysis. High molecular weight genomic DNA was prepared from nuclear pellets as described briefly by Wolgemuth *et al.* (20) and in greater detail by Lewis *et al.* (21). Restriction endonuclease digestions were carried out in buffer systems suggested by the vendors. To ensure complete digestions, proper enzyme-to-DNA ratios were determined empirically. In some cases DNAs were digested once, extracted with phenol/chloroform, centrifuged, concentrated by ethanol precipitation, and digested again with increased amounts of enzyme to verify the restriction patterns initially obtained. Second digestions were routinely carried out in the presence of added control DNA—

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Abbreviations: DHFR, dihydrofolate reductase; kb, kilobase.

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i.e., λ or φX174—to demonstrate the activity of the enzyme.

DNA transfers were performed essentially as described by Southern (22). Prehybridization of the nitrocellulose filters was carried out in 0.9 M sodium chloride/0.09 M sodium citrate, pH 7, 4× Denhardt's reagent, and sheared salmon sperm DNA at 25 μg/ml for 3 hr at 65°C; hybridization then was carried out for 12–16 hr at 65°C in 20 ml of a solution containing 0.6 M sodium chloride/0.06 M sodium citrate, 8 mM EDTA, and sheared salmon sperm DNA at 300 μg/ml with 1–3 × 10^7 cpm of ^{32}P-labeled, nick-translated pDHFR6 (specific activity, 2–4 × 10^8 cpm/μg). Filters then were washed, first in 0.09 M sodium chloride/0.009 M sodium citrate/3.6 mM disodium phosphate/2.4 mM monosodium phosphate/0.018% sodium pyrophosphate for 30 min at 65°C and then in 0.027 M sodium chloride/0.0027 M sodium citrate/1.08 mM disodium phosphate/0.72 mM monosodium phosphate/0.005% sodium pyrophosphate for 30 min at 65°C. After drying at room temperature, the filters were exposed to Kodak X-Omat XAR5 film with intensifying screens for 1–14 days at −80°C.

RESULTS

Distributional Differences of DHFR Poly(A$^+$) RNA in Antifolate-Resistant Chinese Hamster Lung Fibroblast Lines. The results of a RNA transfer analysis of the poly(A$^+$) RNAs from cell lines that overproduced either the M_r 20,000 class or the M_r 21,000 class of DHFR are presented in Fig. 1. As we reported earlier (14) for the prototype M_r 20,000 and M_r 21,000 DHFR overproducing sublines DC-3F/MQ19 and DC-3F/A3, respectively, the pDHFR6 probe hybridizes with three discrete RNA species with molecular sizes of approximately 1,400, 2,200, and 3,300 nucleotides. All three of these mRNAs are present in cell lines that overproduce either the M_r 20,000 or the M_r 21,000 class of the enzyme, and they also are present in the parental line DC-3F. No molecular weight difference is apparent under these electrophoretic conditions between the DHFR mRNAs from parental cells and sublines that overproduce M_r 20,000 DHFR and those from sublines that overproduce M_r 21,000 DHFR.

However, it is important to note that the relative proportion of the three DHFR mRNAs, estimated by the autoradiographic signal of the pDHFR6 probe, varies markedly between the M_r 20,000 and M_r 21,000 DHFR overproducers. As estimated by x-ray film densitometry (data not shown), DHFR poly(A$^+$) mRNAs in the M_r 20,000 DHFR overproducing sublines are distributed with 60–70% as the 1,400-nucleotide species, 15–20% as the 2,200-nucleotide species, and 10–15% as the 3,300-nucleotide RNA. By contrast, the 3,300-nucleotide message predominates in the M_r 21,000 DHFR overproducing sublines and represents 60–70% of the DHFR mRNA, whereas the 1,400-base mRNA accounts for only 10–15%, and the 2,200-base mRNA accounts for 15–20%. These distributional differences are characteristic as well of poly(A$^+$) RNA obtained from polysomal pellets and are invariant from preparation to preparation. Recently (13) we have shown that all three poly(A$^+$) DHFR mRNA species can be translated *in vitro*. As expected, all three DHFR mRNAs from cell lines that overproduce M_r 20,000 DHFR translate to yield M_r 20,000 DHFR, whereas all three DHFR mRNAs from cell lines that overproduce M_r 21,000 DHFR translate to M_r 21,000 DHFR.

Restriction Enzyme Analysis of DHFR Gene Sequences in DHFR Overproducing Cell Lines. Because a selective amplification of polymorphic DHFR genes in the DC-3F genome conceivably might account for the correlation of DHFR molecular weight with the DHFR mRNA size distribution patterns in the various overproducing cell lines, we analyzed restriction

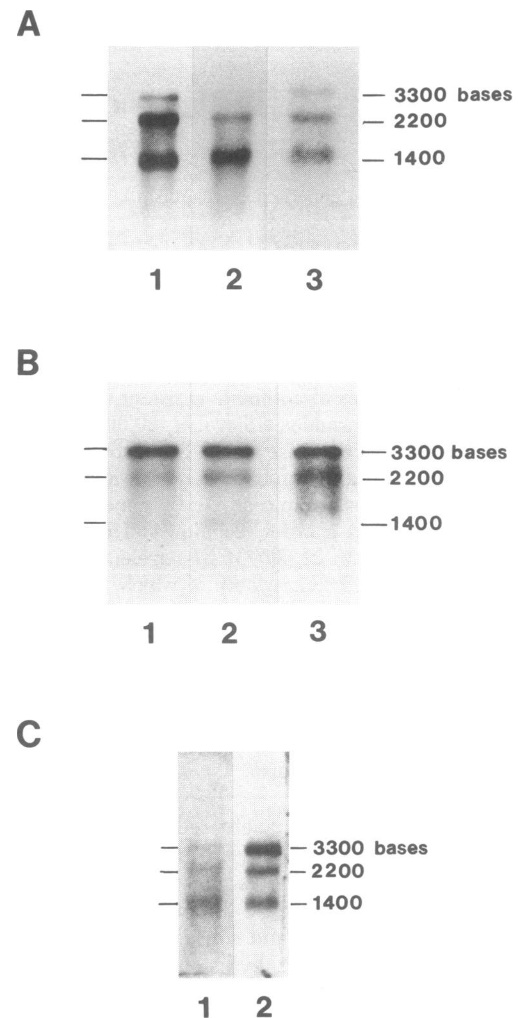

FIG. 1. RNA transfer analysis of mRNA from Chinese hamster lung cells. Polysomal poly(A$^+$) RNA was denatured in a buffer containing 50% deionized formamide/2.2 M formaldehyde at 65°C for 10 min; it then was quickly cooled on ice and electrophoresed for 16 hr at 40 V in a 1.5% agarose gel containing 2.2 M formaldehyde. Transfer of RNA to diazobenzyloxymethyl-paper or nitrocellulose paper and hybridization with nick-translated pDHFR6 was as described. In each case, the amount of RNA loaded per well depended upon the amount of DHFR produced by the particular subline and varied between 1 μg for sublines overproducing DHFR ≥100-fold and 15 μg for the control line DC-3F. In the following listing the value in parenthesis designates the increase in DHFR activity relative to the control cell line DC-3F. (*A*) Sublines that overproduce M_r 20,000 DHFR. Lane 1, DC-3F/MQ8 (×144), 1 μg; lane 2, DC-3F/A1 (×21), 5 μg; lane 3, DC-3F/A55 (×4.6), 5 μg. (*B*) Sublines that overproduce M_r 21,000 DHFR. Lane 1, DC-3F/A75 (×121), 1 μg; lane 2, DC-3F/MQ29 (×122), 1 μg; lane 3, DC-3F/MQ20 (×49), 5 μg. (*C*) Control cell lines DC-3F. Lane 1, RNA representative of DC-3F (×1) cells grown in November 1979, 10 μg; lane 2, RNA representative of DC-3F/30 (×1), a clone of DC-3F grown in December 1981, 10 μg.

enzyme-generated Southern blot patterns of genomic DHFR sequences from the M_r 20,000 and M_r 21,000 DHFR overproducing sublines DC-3F/MQ19 and DC-3F/A3, respectively, by using pDHFR6 as the hybridization probe. Whereas the enzymes *Bam*HI, *Bcl* I, *Pvu* II, *Pst* I, and *Eco*RI revealed no difference between DNA from the M_r 20,000 and M_r 21,000 DHFR overproducers, the enzyme *Hin*dIII, as shown in Fig. 2, conveniently distinguished the amplified M_r 20,000 DC-3F/MQ19 DHFR DNA from the amplified M_r 21,000 DC-3F/A3 DHFR DNA. A subsequent survey of the other 14 drug-re-

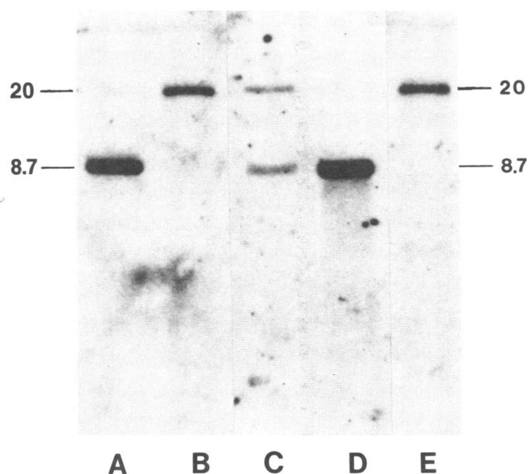

FIG. 2. Southern blot analysis of genomic DNAs from Chinese hamster lung cells that overproduce M_r 20,000 or M_r 21,000 DHFR. High molecular weight genomic DNA was digested with *Hin*dIII at an enzyme-to-DNA ratio of 5–10 units/μg for 18 hr at 37°C. Samples then either were adjusted to a composition of 40 mM Tris, pH 7.9/7 mM sodium acetate/1 mM EDTA/2% Ficoll/0.1% bromophenol blue and electrophoresed directly or were extracted in phenol/chloroform, precipitated in ethanol, and then solubilized in the same buffer. Electrophoresis was for 16–20 hr in 0.8% agarose at 50 V. The molecular weight marker used (but not shown) was *Hin*dIII cut λ DNA. Transfer of DNA to nitrocellulose paper and hybridization with nick-translated pDHFR6 was as described. Numbers shown are in kb. Values in parentheses are as in Fig. 1. Lane A, DC-3F/MQ19 (×151) M_r 20,000 overproducer, 1 μg; lane B, DC-3F/A3 (×170) M_r 21,000 overproducer, 1 μg; lane C, DC-3F (×1), 10 μg; lane D, DC-3F/MQ8 (×144) M_r 20,000 overproducer, 1 μg; lane E, DC-3F/Ab17 (×281) M_r 21,000 overproducer, 1 μg.

sistant sublines available to us—part of the results of which are shown in Fig. 2 lanes D and E and Fig. 3—permits a correlation to be drawn among *Hin*dIII genomic DNA pattern, DHFR mRNA size distribution profile, and the molecular weight class of DHFR overproduced, such that cell lines that overproduce the M_r 20,000 enzyme class contain a predominant 1,400-base DHFR mRNA and amplify an 8.7-kilobase (kb) *Hin*dIII genomic DNA fragment, whereas cell lines that overproduce the M_r 21,000 enzyme class contain a predominant 3,300-base DHFR mRNA and amplify a 20-kb *Hin*dIII fragment.

Restriction Enzyme Analysis of DHFR Gene Sequences in the DC-3F Parent Cell Line. Using the *Hin*dIII enzyme as a diagnostic reagent, we analyzed the DHFR DNA sequences of the drug-sensitive parental cell line DC-3F. We expected, if DHFR molecular weight and DHFR mRNA size distribution were phenotypic expressions of polymorphic DHFR genes, to detect both an 8.7-kb fragment characteristic of the amplified M_r 20,000 DHFR gene and a 20-kb fragment characteristic of the amplified M_r 21,000 DHFR gene. The results of a Southern blot analysis of genomic DNA from the DC-3F cell line, shown in Fig. 2, reveal both the 8.7-kb and 20-kb bands, as predicted. To establish strictly that both the 8.7-kb and 20-kb DHFR DNA sequences are present in the genome of a single DC-3F cell, the DC-3F population was subcloned by serial dilution and the DHFR genomic sequences of six randomly selected subclones were analyzed. The results (not shown) confirm that both an 8.7-kb and a 20-kb *Hin*dIII band are present in the DC-3F cell genome.

Persistence of the Unamplified DHFR Allele in DHFR Overproducing Lines. Longer exposures of the Southern blots in Fig. 2 reveal a faint 20-kb band in M_r 20,000 DHFR overproducing cell lines and, conversely, a faint 8.7-kb band in M_r 21,000 DHFR overproducing cell lines, suggesting the continued presence of an unamplified polymorphic DHFR gene in these lines. We analyzed the *Hin*dIII restriction patterns of the genomic DNAs obtained from sublines DC-3F/MQ10, DC-3F/MQ31, and DC-3F/A55 with DHFR gene amplifications of 2- to 5-fold (21) to demonstrate convincingly that the alternate unamplified DHFR gene is retained while the other is amplified. As shown in Fig. 3, the 20-kb *Hin*dIII band in the M_r 21,000 DHFR overproducing MQ10 and MQ31 cell lines is intensified relative to the 8.7-kb band; conversely, the 8.7-kb band is intensified relative to the 20-kb band in the M_r 20,000 DHFR overproducing DC-3F/A55 line. These results clearly suggest that an amplification either of the 8.7-kb or the 20-kb genomic DHFR sequence does not proceed through the loss or apparent modification of the alternate DHFR sequence.

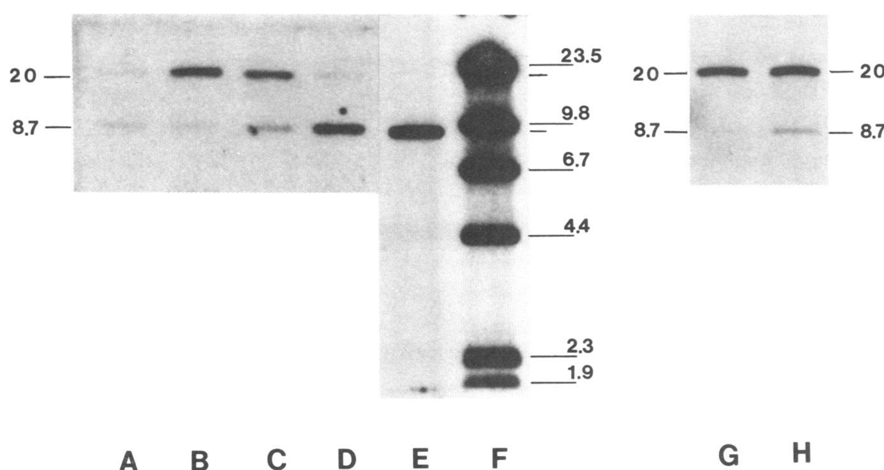

FIG. 3. Demonstration that drug-resistant sublines amplify one DHFR gene while retaining single copy amounts of another. High molecular weight genomic DNA was digested with *Hin*dIII and analyzed by Southern blotting techniques by using pDHFR6 as a probe (see Fig. 2). *Hin*dIII-cut λ DNA served as an internal marker in these experiments and was hybridized by adding a trace amount of nick-translated λ DNA to the hybridization mixture. DNA loads and autoradiographic exposure times were adjusted to optimize visualization of the nonamplified genes. Numbers shown are in kb. Values in parentheses are as in Fig. 1. Lane A, DC-3F (×1) synthesizes both M_r 20,000 and M_r 21,000 DHFR, 10 μg; lane B, DC-3F/MQ10 (×2.9) M_r 21,000 overproducer, 10 μg; lane C, DC-3F/MQ31 (×3.9) M_r 21,000 overproducer, 10 μg; lane D, DC-3F/A55 (× 4.6) M_r 20,000 overproducer, 10 μg; lane E, DC-3F/A1 (×21) M_r 20,000 overproducer, 5 μg; lane F, *Hin*dIII-cut λ DNA marker; lane G, DC-3F/A75 (×122) M_r 21,000 overproducer, 1 μg; lane H, DC-3F/MQ29 (×122) M_r 21,000 overproducer, 1 μg.

DISCUSSION

We interpret the results presented above to argue for the presence in the DC-3F genome of at least two polymorphic DHFR genes, distinguished by the molecular weight of the protein they encode, by the size distribution of their polyadenylylated polysomal RNA transcripts, and by nucleotide sequence as defined by *Hin*dIII restriction enzyme analysis. Though genetic polymorphisms resulting in protein structure or mRNA sequence alterations (or both) are well known (23–26), we believe that this definition of RNA distributional difference as an aspect of gene polymorphism has not previously been reported. At present we have no sequence data that would relate the protein, mRNA, and DNA results to a single nucleotide sequence change or variation. Though the nucleotide sequences putatively involved with poly(A) addition and transcription termination lie in 3′ untranslated nucleotide sequences, it remains a formal possibility that presently undefined variations in coding nucleotides can account simultaneously for the translational and transcriptional differences we attribute to M_r 20,000 DHFR and M_r 21,000 DHFR gene sequences. Because our analysis of the genomic DHFR sequences of M_r 20,000 DHFR or M_r 21,000 DHFR overproducing line has relied on a blotting probe—pDHFR6, which contains only 40% of the sequences of the smallest DHFR mRNA—we have surveyed with the six restriction enzymes used here only a small fraction of the DHFR gene nucleotides and, therefore, are unable to estimate the sequence divergence of the proposed M_r 20,000 DHFR and M_r 21,000 DHFR genes. However, we have recently obtained full coding sequence cDNAs for mRNAs from both the DC-3F/MQ19 and DC-3F/A3 cell lines and have cloned them under conditions in which their respective DHFRs are expressed in *Escherichia coli*. Determination of the nucleotide sequence of these double-stranded cDNAs should allow a direct estimate of the degree of divergence of Chinese hamster DHFR coding sequences.

Our demonstration of the selective amplification of polymorphic DHFR genes is consistent with previous cytogenetic data. The earlier description of cytogenetic alterations of chromosome 2 [the presumed location of the Chinese hamster DHFR gene (27)] by Biedler *et al.* (11) emphasized importantly that only one of two homologs was involved in the amplification process—an observation recently extended by us to include drug-resistant sublines exhibiting low-level (i.e., 2- to 5-fold) DHFR gene amplification (21). Additionally, we have shown that of 16 DHFR overproducing sublines analyzed, none overproduces more than one molecular weight class of DHFR. If the DC-3F cell line is homozygous at the DHFR locus and if, as a result of a gene duplication, both a M_r 20,000 and a M_r 21,000 DHFR gene are present in tandem on each homolog, then amplification on either homolog could involve either one of the two DHFR genes or both of them. However, this would require a strict regulation of sequence involvement in the amplification process, for at no time have we observed both the M_r 20,000 and M_r 21,000 DHFR genes coamplified (Figs. 2 and 3). Because the size of the DHFR amplification unit in these cells has been estimated at 350–700 kb (21), we consider it highly unlikely that coamplification of both genes would be consistently precluded. Alternatively, therefore, we suggest that the DC-3F line is heterozygous at the DHFR locus with the M_r 20,000 DHFR gene on one homolog of chromosome 2 and the M_r 21,000 DHFR gene on the other. Hence, independent amplification of either allele can occur with the resulting overproduction of either molecular weight class of DHFR.

We have recently obtained evidence that both alleles are expressed in the DC-3F genome. We have analyzed the DHFR protein and mRNA pattern in various clones from the DC-3F subcloning mentioned earlier and have found both the M_r 20,000 and M_r 21,000 DHFR classes present (13) and a poly(A^+) RNA profile that resembles a superposition of a M_r 21,000 DHFR mRNA pattern on a M_r 20,000 DHFR pattern (Fig. 1*C*). This recent result, although consistent with our genetic interpretation, is at variance with an earlier report (14) in which the DHFR mRNA pattern of DC-3F resembled that expected of a cell expressing only the M_r 20,000 DHFR gene. To date, this variable result stands as unique in our experience. We have repeatedly analyzed mRNA obtained from overproducing cell lines over long periods of time and have yet to see variation in DHFR mRNA size distribution. However, the variation in DC-3F mRNA distribution is consistent with parallel variations in the predominant molecular weight class of DHFR synthesized by the DC-3F parent cell line (13).

Though we do not understand the nature of this control cell variation, genomic DNA *Hin*dIII blotting profiles of DC-3F cells have been invariant over the course of these studies, ruling out random loss of DHFR genes as the cause of the variable DC-3F phenotype. Alternatively, it is possible that in the absence of suitable selective pressure, the relative expression of alleles in tissue culture cells may simply drift or be affected by subtle changes in culturing conditions or cell passage number. It is also conceivable that the expression of the M_r 20,000 and M_r 21,000 DHFR genes is variously regulated through the cell cycle and that our cell harvests, although intended as mid- to late-logarithmic, vary sufficiently to favor the expression of one allele or the other.

Regardless of how this issue is resolved, we continue to favor the interpretation that the DHFR locus of the DC-3F cell line is polymorphic and that this polymorphism involves both RNA transcription and DHFR protein structure. Despite these differences, both genes are capable of gene amplification. We are not certain whether the predominant amplification of the M_r 21,000 DHFR allele in the DC-3F system is statistically significant, though it is formally possible that some sequences are more amenable to the process of amplification and that the M_r 21,000 DHFR allele is associated with such favored sequences. Characterization of cloned DHFR sequences in λ-phage vectors should permit analysis of these polymorphic genes to extend to the flanking regions in which sequences important to amplification may lie.

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