Gene Amplification Accompanies Low Level Increases in the Activity of Dihydrofolate Reductase in Antifolate-resistant Chinese Hamster Lung Cells Containing Abnormally Banding Chromosomes

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ABSTRACT Three independently-derived, antifolate-resistant Chinese hamster lung cell lines that exhibit low level increases in dihydrofolate reductase (DHFR) activity, i.e., three- to fivefold vs. controls, have been compared with drug-sensitive cells to determine relative DHFR gene content. With a solution hybridization technique that makes use of genomic DNA and a cloned double-stranded Chinese hamster DHFR cDNA probe, it has been found that the enzyme activity increases are associated with an approximately proportionate amplification of DHFR genes. Trypsin-Giemsa staining techniques and hybridizations *in situ* further show that the amplified DHFR genes are located within abnormally banding regions along chromosome 2q and also suggest that, in each subline, only one chromosome 2 homolog is initially involved in the amplification process.

The observation that amplification of dihydrofolate reductase (DHFR) genes accompanies the overproduction of this enzyme in cultured mammalian cells challenged with the antifolate drugs methotrexate and methasquin has been reported by several investigators (1–6). Although primarily studied in antifolate-resistant cell lines displaying high levels of DHFR overproduction (e.g., 50- to 300-fold), where the correlation between the amount of enzyme overproduced and the degree of gene amplification has been shown to be essentially proportional, the question of whether low level overproduction of the enzyme (e.g., three- to fivefold) is also associated with amplification of DHFR genes remains open. While there appears to be no compelling reason to evoke an amplification mechanism as the mode whereby antifolate-resistant cells maintain a low level of dihydrofolate reductase overproduction, indirect evidence that this might be the case has come from cytological studies performed by Biedler and her colleagues (7–10) with a number of Chinese hamster lung cell lines varying in DHFR activity from 2- to 300-fold.

Initially, using conventional staining techniques (7) and then trypsin-Giemsa banding methods (8) to analyze the karyotypes of a large series of independently derived Chinese hamster sublines, selected with methotrexate or methasquin, respectively, it was found that specific chromosome abnormalities, preferentially associated with one homolog of the chromosome 2 pair, were consistently associated with DHFR overproduction. In all drug-resistant cell lines with greater than 100-fold increases in DHFR activity, the chromosome abnormalities were manifested as long, usually terminal segments on chromosome 2q which failed to band in the cross-striational pattern expected with trypsin-Giemsa techniques and appeared as medium gray, homogeneously staining regions, HSRs (9).

In those sublines with less than 100-fold increases in DHFR activity, the chromosomal abnormalities revealed themselves not as HSRs but as interruptions or disruptions in the normal Giemsa banding pattern of chromosome 2q with apparent additions or substitutions by novel, abnormally banded segments characteristic of each subline. These regions, like HSRs, have been termed "abnormally banding regions" and have been suggested to be cytological manifestations of gene amplification as originally proposed (10) and later demonstrated (2) for HSRs. Recent results of Melera et al. (3) documenting a 20-fold DHFR gene amplification in one of these sublines, DC-3F/MQ20 (a 50-fold DHFR overproducer), and *in situ* hybridization data which indicate that the abnormally banded regions in sublines DC-3F/MQ20 and DC-3F/A1 (a 20-fold

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DHFR overproducer) contain DHFR genes (J. L. Biedler, P. W. Melera, and B. A. Spengler, manuscript in preparation) provide additional evidence that these regions do, in fact, represent sites of gene amplification. It is possible, however, in the cases where the increases in DHFR activity are small, i.e., 2.9-, 3.9-, and 4.9-fold in sublines DC-3F/MQ10, DC-3F/MQ31, and DC-3F/A55, respectively, that the abnormally banding regions develop not from amplification events but, rather, from chromosomal breaks and rearrangements which situate a single DHFR gene in novel contexts of flanking nucleotide sequences. Such DHFR gene relocations might permit increased transcriptional activity at the new loci, thus allowing low level DHFR overproduction without increases in DHFR gene number. It was of interest, therefore, to establish whether DHFR gene amplification could be demonstrated in drug-resistant cell lines which exhibited low level increases in DHFR activity and whether the abnormally banding regions in the chromosomes of these cells contained DHFR genes.

MATERIALS AND METHODS

Cell Lines and Chromosome Analysis

Derivation and phenotypic characterization of the three antifolate-resistant sublines included in this study are detailed elsewhere (7, 8). DC-3F/MQ10 and DC-3F/MQ31 were selected by stepwise increases in drug to a final concentration of 1 μg/ml of methasquin and exhibit a 117- and 133-fold increase in resistance to drug. DC-3F8/A55 was selected at a final concentration of 5 μg/ml of methotrexate and exhibits a 4,455-fold increase in resistance to that antifolate. Results of karyotype analysis of the three sublines included in this study as well as of 13 additional independently-derived antifolate-resistant Chinese hamster lung cell lines with higher DHFR levels have been reported (9). Metaphase cells were prepared and stained by trypsin-Giemsa banding methods.

DNA Isolation and Purification

DNAs were isolated from nuclear pellets obtained from the three antifolate-resistant sublines included in this study, essentially as described by Melera et al. (3). Nuclei were suspended in a buffer of 10 mM Tris-HCl, pH 7.4, 400 mM NaCl, 10 mM EDTA containing 100 μg/ml Proteinase-K, lysed by the addition of SDS to 0.5% and incubated for 24–36 h at 37°C with gentle agitation. DNA solutions were extracted twice with phenol:chloroform:isoamyl alcohol and high molecular weight DNA obtained by spooling. Spooled DNA was pressed free of excess ethanol, resuspended in a buffer of 10 mM Tris HCl (pH 7.4), 20 mM EDTA, and incubated for 3 h at 37°C with 50 μg/ml RNase A. The DNA solution was next digested with 100 μg/ml Proteinase K for 3 h at 37°C after the addition of NaCl to 400 mM. This digest was then extracted twice with phenol:chloroform:isoamyl alcohol and, after the addition of 2.5 vol of ethanol, the high molecular weight DNA was collected again by spooling, dried under vacuum, and resuspended in 100 mM NaAc, pH 7.0.

DNA Shearing and Chelex Treatment

High molecular weight DNA was reduced to 350–400 base pair (bp) fragments by shearing in a Virtis Model 60 homogenizer essentially as described by Britten et al. (11). DNA solutions in 100 mM $NaAc^-$ pH 7.0 were adjusted to 66% vol/vol glycerol and sheared at 54,000 rpm for 30 min at 0–4°C. After the addition of $NaAc^-$ to 0.3 M, DNA was precipitated from 2–5 vol of 95% ethanol at −20°C for 18–24 h, collected by centrifugation, and sized by alkaline agarose gel electrophoresis, according to McDonnell et al. (12), using Hae III digests of φX174 as molecular weight markers. DNA preparations were then resuspended into 100 mM $NaAc^-$, pH 7.4, and treated with Bio-Rad Chelex 100 (Bio-Rad Laboratories, Richmond, CA) by batch procedures. Up to 20 mg of DNA were treated with 2.5 ml of packed Chelex previously equilibrated to pH 7.4 in 100 mM $NaAc^-$.

pDHFR6 Insert Purification and Nick Translation

The 650 bp DHFR-specific insert of pDHFR6 (13), cloned into pBR322 with Eco RI and Sal I linkers, was isolated by electrophoresis through native 1.5% agarose gels after the digestion of pDHFR6 with Eco RI and Sal I enzyme. Insert DNA was located in the ethidium bromide-stained gel (5 μg/ml) by U.V. illumination, and a section of the gel containing the insert was enclosed in dialysis tubing and electroeluted over a 3-h period. DNAs were concentrated by DEAE-Sephacel chromatography and ethanol precipitation. The recombinant insert was nick-translated with ^{32}P dXTPs essentially as described by Rigby et al. (14). Specific activities ranged from 3 to 7×10^7 cpm/μg, depending on the DNA preparation. DNAs radiolabeled by this protocol had an average molecular size of 250 bp as determined by alkaline gel electrophoresis (12) with Hae III digests of φX174 as molecular weight standards. Snapback DNA generated by *Escherichia coli* Pol I during nick translation was removed as follows. The labeled probe was heat-denatured, reassociated to a C_ot of 10^{-4} and fractionated by hydroxylapatite (HAP) chromatography. The sequences remaining single-stranded through this period were recovered in 0.12 M $NaPO_4$ and were capable of reassociation to >92% at C_ot 10^{-1} when reanalyzed.

Reassociation Analysis and HAP Chromatography

Sheared genomic DNAs were resuspended in a hybridization buffer of 0.14 M $NaPO_4$, pH 6.8, 0.1% SDS as described by Sambrook et al. (15). 250 μg of genomic DNA was combined with 0.05 ng of pDHFR6 insert (3,500 cpm ^{32}P) in a 100 μl reaction and heat denatured by boiling. Reactions were brought to 60°C, adjusted to 1.0 M NaCl, and incubated at 60°C. At appropriate times, 25-μl aliquots of the main reaction were withdrawn, diluted to 1.0 ml in 0.12 M $NaPO_4$, quick-frozen, and held at −20°C until assay by HAP chromatography. Individual samples were chromatographed on Bio-Rad HTP hydroxyapatite columns prepared in Pasteur pipettes plugged with silanated glass wool. Multiple samples were processed simultaneously in a circulating water bath maintained at 60°C. Single-stranded DNAs were eluted in 5-column volumes of 0.12 M $NaPO_4$. Double-stranded DNAs were eluted in 5-column volumes of 0.5 M $NaPO_4$. Aliquots of both fractions were mixed with 2.0 vol of Biofluor (New England Nuclear, Boston, MA), cooled to 4°C, and quantified by scintillation counting.

In Situ Hybridization

Metaphase plates for hybridization studies were prepared by standard procedures after exposure to 0.1–0.5 μg/ml colcemid for 4–6 h. After digestion with ribonuclease A, cellular DNA was denatured in 0.07 N NaOH, twice SSC (0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0), for 2–3 min at ambient temperature. The hybridization mixture contained 50% formamide, four times SSC, pH 7.0, 10% sodium dextran sulfate 500, once Denhardt's reagent (0.02% wt/vol bovine serum albumin, polyvinyl pyrrolidone, and Ficoll, M_r = 400,000), 0.1 M potassium iodide, 100 μg/ml denatured salmon sperm DNA, and either 35–70 ng/ml or 7 μg/ml of nick-translated, [^{125}I]-labeled pDHFR6 probe (sp act 2 to 9×10^8 DPM/μg). Slides with 8–10 μl of the hybridization mixture under 18-mm^2 cover glasses were incubated in moist chambers (16) at 37°C for 6 to 18 h. Preparations were washed at 37°C in three changes of four times SSC, pH 7.0, for 10 min each, in once Denhardt's solution for 1 h, and in six additional rinses of four times SSC for a total of 18–20 h, dehydrated in ethanol, and air-dried. Slides were dipped in Kodak NTB-2 liquid emulsion diluted 1:1 with water, exposed at 4°C for 7 d to 4 mo, developed in Kodak D-19 developer, and stained in a 1:50 dilution of Giemsa's. Additional experimental details are given elsewhere (J. L. Biedler, P. W. Melera, and B. A. Spengler, manuscript in preparation).

RESULTS

Representative G-banded metaphase cells of the antifolate-resistant sublines DC-3F/MQ10, DC-3F/MQ31, and DC-3F/A55 with abnormally banding regions on chromosome 2q are shown in Fig. 1. These cell lines, overproducing DHFR 2.9-, 3.9-, and 4.9-fold, respectively, have abnormal regions which are 0.74, 2.2, and 0.62 times, respectively, the length of the short (*p*) arm of the affected number 2 chromosome (9). The abnormal banding pattern can be seen to be highly cell line-specific (Fig. 1). Although these sublines are marked by additional, apparently random, structural chromosome rearrangements, as previously detailed (7–9), none of the latter resemble the long chromosomal regions with clearly abnormal, unidentifiable bands preferentially located on chromosome 2q. It should be noted that sublines DC-3F/MQ10 and DC-3F/MQ31 are near-tetraploid, whereas subline DC-3F/A55 is near-diploid. Of interest here is the observation that two of the four chromosome 2 homologs in the DC-3F/MQ31 subline contain abnormally banding regions, whereas only one of the

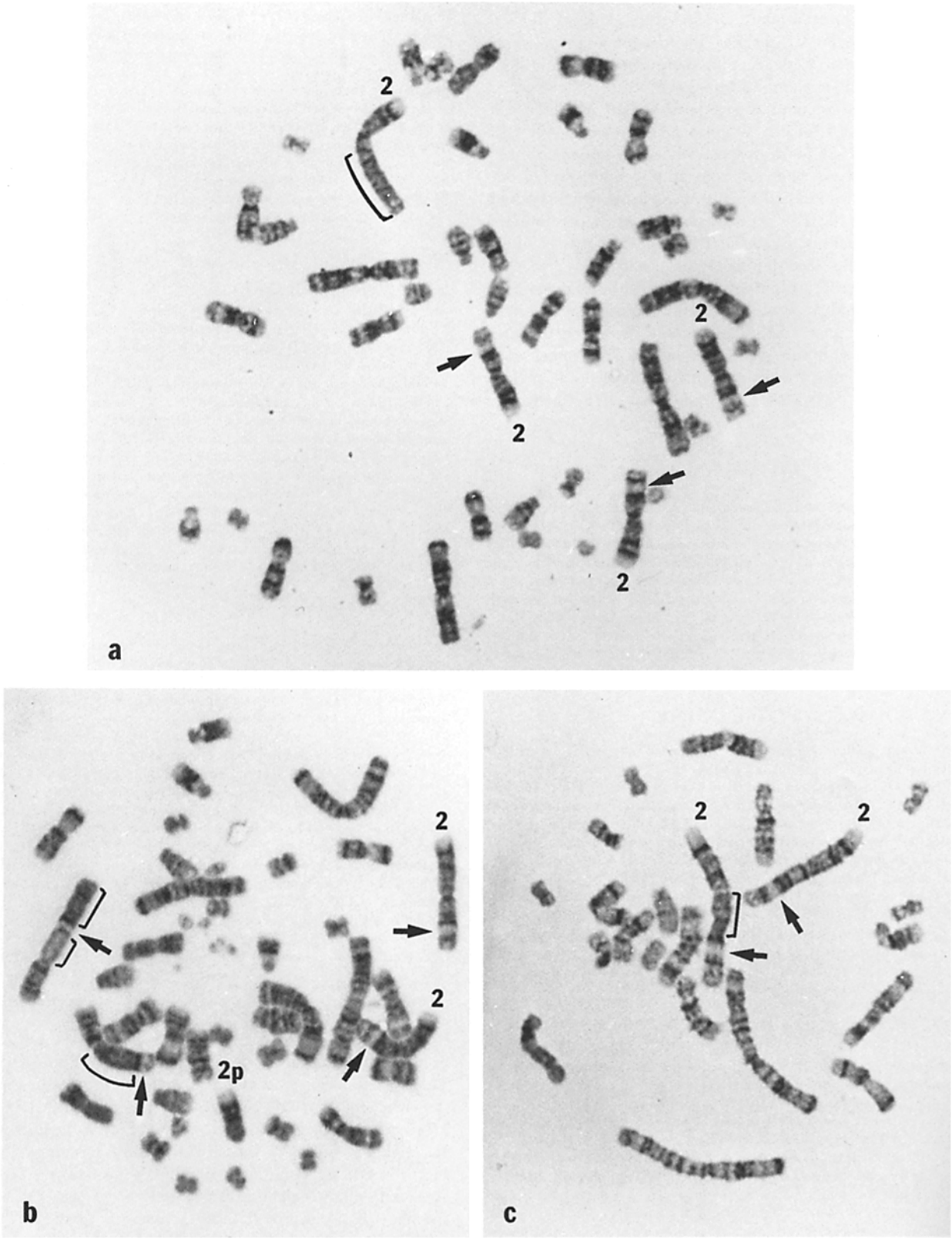


FIGURE 1 G-banded metaphase cells. Abnormally banding regions are indicated by brackets. Arrows indicate the wide, palely staining band 8 of chromosome 2q (9). (*a*) Subline DC-3F/MQ10. Cells have a modal chromosome number of 42 (8) and consistently contain three normal chromosome 2's, as indicated, and one abnormal homolog with an abnormally banding region comprising most of the 2q arm. (*b*) Subline DC-3F/MQ31. The modal chromosome number is 39 or 40 (8). Cells have two normal chromosome 2's and a chromosome consisting of a normal *2p* arm. A fourth *2p* arm is not present. The other 2q arms are recognizable as distinctive segments comprising bands 7 and 8 (9), bound on one or both ends by abnormally banding regions, and translocated to a normal 5q. (Chromosome 5 was designated a 6 in our previous publications.) Almost every cell has two versions of the same abnormal chromosome. In this cell the distal half of the longer chromosome with the abnormally banding region is identical to the abnormal region of the shorter chromosome. This cell has possible double minute chromosomes (at center of cell), a rare finding in antifolate-resistant Chinese hamster lung cells. (*c*) Subline DC-3F8/A55. This line has a modal chromosome number of 22 and an abnormally banding region located interstitially on the long arm of a single chromosome 2. × 1,500.

four does so in DC-3F/MQ10. As described in the legend to Fig. 1, the two abnormal chromosomes in DC-3F/MQ31 are essentially identical. Only one chromosome 2 homolog in the DC-3F/A55 subline contains an abnormally banding region, just as observed in all the other diploid lines we have examined. Since none of the abnormally banding regions found among our 17 drug-resistant sublines have the same appearance, it is highly unlikely that two of the four chromosome 2 homologs in subline DC-3F/MQ31 independently acquired identical abnormally banding regions. We suggest, therefore, that the two abnormally banded homologs are present because the cell line acquired the abnormally banding region before tetraploidization occurred. It also follows that the abnormally banding region on the single chromosome 2 homolog in subline DC-3F/MQ10 must have occurred after tetraploidy was established, since, had it occurred before, two homologs of the tetraploid cell would be affected. The discriminatory nature of the mechanism in these cell lines which apparently limits involvement of more than one chromosome 2 homolog in the acquisition of abnormally banding regions is not understood.

To establish whether DFHR gene amplification had occurred in the antifolate-resistant sublines with small increases in DHFR activity, the 650 bp DHFR specific double-stranded DNA insert of the recombinant plasmid pDHFR6 was isolated, labeled in vitro by nick translation and reacted in solution hybridization with genomic DNAs from either the control DC-3F cell line or the cell lines DC-3F/MQ10, DC-3F/MQ31, and DC-3F/A55. The kinetics of hybridization were analyzed mathematically according to the equations detailed by Sambrook et al. (15).

The self-reassociation of the labeled DHFR probe was first established by hybrid formation in the presence of sheared salmon sperm DNA. 250 μg of salmon sperm DNA was combined with 0.05 ng of probe, heat-denatured and hybridized at 60°C. At various times aliquots of the main reaction were withdrawn for analysis of hybrid formation by HAP chromatography. At each time point a solution of the equation $1/f_{ss} = t/t_{1/2} + 1$ (where f_{ss} is the fraction of probe remaining single-stranded and t is the time of reannealing) gave a value for $t_{1/2}$, the time required for one half of the probe sequences to be in DNA duplex. Determination of the $t_{1/2}$ at each time point throughout the course of the reaction yielded an average $t_{1/2}$ value of 6.33 ± 0.67 h (see Table I, Probe alone). When the labeled probe was reacted in the presence of genomic DNA containing homologous DHFR sequences, its reassociation was accelerated to a degree dependent on the concentration of homologous sequences supplied in the genomic DNA. As expected, the reassociation of the DHFR probe was accelerated in the presence of genomic DNA from control drug-sensitive DC-3F cells, as reflected by the reduced average $t_{1/2}$ value of 2.67 ± 0.19 h (Table I). This value for $t_{1/2}$ in the presence of DC-3F was then compared with the $t_{1/2}$ values determined by the reassociation of the probe in the presence of the genomic DNAs from each of the three DHFR-overproducing sublines. In each case (Table I), the reassociation rate of the labeled DHFR probe in the presence of resistant subline DNA was accelerated relative to its rate of reassociation in the presence of DC-3F DNA, thus demonstrating the presence of increased numbers of DHFR genes in drug-resistant cells. The $t_{1/2}$ values were reduced to 0.91 ± 0.14, 0.82 ± 0.13, and 1.26 ± 0.11 h by reaction with DC-3F/MQ10, DC-3F/MQ31, and DC-3F/A55 genomic DNA, respectively. These accelerated kinetics were revealed graphically by an increase in the slope of the line drawn by a plot of the $1/f_{ss}$ values for each time point for each DNA against the $1/t_{1/2}$ values established at each time point for the reassociation of the probe sequences in the presence of salmon sperm DNA (Fig. 2). Using the $t_{1/2}$ values established in the presence of DC-3F DNA, the relative increases in DHFR gene number in the DC-3F/MQ10, DC-3F/MQ31, and DC-3F/A55 cell lines were estimated by the ratio $t_{1/2}$ sensitive cell DNA/$t_{1/2}$ resistant cell DNA, and are presented in Table II. As with our previous results using sublines overproducing DHFR 100-fold or more (3), the relative increases in DHFR gene copy number in these three sublines were approximately equal to the relative increases in their DHFR activity.

Identification of the chromosomal sites of amplified DHFR genes in the DC-3F/MQ10 and DC-3F8/A55 sublines was made by hybridization *in situ* using nick-translated pDHFR6 as probe (Fig. 3). In each case the autoradiograms showed clusters of grains specifically associated with those regions of chromosome 2q known to be abnormally banding (see Fig. 1), while the remainder of the chromosomes were free of consistent grain clusters. Although a more quantitative treatment of these data will appear elsewhere (J. L. Biedler, P. W. Melera, and B. A. Spengler, manuscript in preparation), they serve here to show that the majority, if not all, of the amplified DHFR genes demonstrated to exist in these sublines (Table II) are contained within the abnormally banding regions of chromosome 2q. Assuming that the diploid parental cell line DC-3F contains two copies of the DHFR gene, one on each chromosome 2 homolog to which the native Chinese hamster DHFR gene has tentatively been assigned (17), it can be determined from the data of Table II that sublines DC-3F/MQ10 and DC-3F/MQ31 each contain twelve copies of the DHFR gene and that subline DC-3F/A55 contains four copies. The abnormally banding region in the tetraploid subline DC-3F/MQ10, therefore, contains nine DHFR genes with the remaining three copies of the gene present at one copy per normal homolog.

TABLE I

Renaturation of ^{32}P-labeled Recombinant Insert of pDHFR 6 in the Presence of Genomic DNA from the Control DC-3F Cell Line or the Resistant Lines DC-3F/MQ10, DC-3F/MQ31, and DC-3F8/A55

	$t^1/_2$*						
Time (h)‡	1	2	3	4	5	7	Average value
Probe alone	5.44 ± 0.44	5.71	6.63 ± 0.51	6.55	6.95 ± 0.29	6.70	6.33 ± 0.67
DC-3F	2.50 ± 0.0	2.35	2.85 ± 0.15	2.79	2.82 ± 0.12	2.72	2.67 ± 0.19
DC-3F/MQ10	0.68 ± 0.04	0.81	0.97 ± 0.03	1.00	1.06 ± 0.11	0.95	0.91 ± 0.14
DC-3F/MQ31	0.67 ± 0.04	0.70	0.91 ± 0.09	0.83	0.95 ± 0.22	0.86	0.82 ± 0.13
DC-3F8/A55	1.06 ± 0.02	1.28	1.30 ± 0.0	1.33	1.29 ± 0.04	1.33	1.26 ± 0.11

* $t^1/_2$ = the time required for the DHFR probe to be 50% reassociated by reaction with genomic driver DNA. Values for $t^1/_2$ are computed according to the equation $1/f_{ss} = t/t^1/_2 + 1$, where f_{ss} is the fraction of probe remaining single-stranded and t is the time of sample incubation.

‡ Each 1-, 3-, and 5-h value represents the average from two separate experiments. The 2-, 4-, and 7-h values represent single determinations.

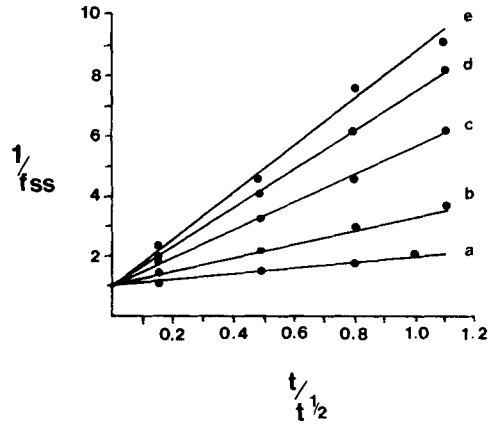


FIGURE 2 Renaturation of ^{32}P-labeled recombinant insert of pDHFR6 in the presence of genomic DNA from the DC-3F control line or the resistant lines DC-3F/MQ10, DC-3F/MQ31, and DC-3F8/A55. 0.05 ng recombinant insert of pDHFR6 nick-translated to a specific activity of 7.7×10^7 ^{32}P cpm/μg was heat-denatured in the presence of 250 μg genomic DNA, cooled to 60°C and hybridized for various times in a buffer of 0.14 M $NaPO_4$, 1.0 M NaCl, 0.1% SDS in a total reaction volume of 100 μl. Aliquots of the main reaction were withdrawn at the appropriate times and quick-frozen in 1.0 ml 0.12 M $NaPO_4$ and held at −20°C until fractionation by HAP chromatography. Aliquots of both HAP fractions were mixed with Biofluor and quantified by scintillation counting. The renaturation of the probe alone was conducted in the presence of 250 μg salmon sperm DNA sheared to 400 bp. All genomic DNAs were sheared to 400 bp before analysis. The data shown was obtained from Table I, as described in the text. (*a*) Probe alone. (*b*) DC-3F. (*c*) DC-3F8/A55. (*d*) DC-3F/MQ10. (*e*) DC-3F/MQ31.

TABLE II

*Genomic DHFR Gene Complements of the Resistant Cell Lines DC-3F/MQ10, DC-3F/MQ31, and DC-3F8/A55 Estimated by the Ratio: t½ Sensitive Cell DNA */ t½ Resistant Cell DNA*

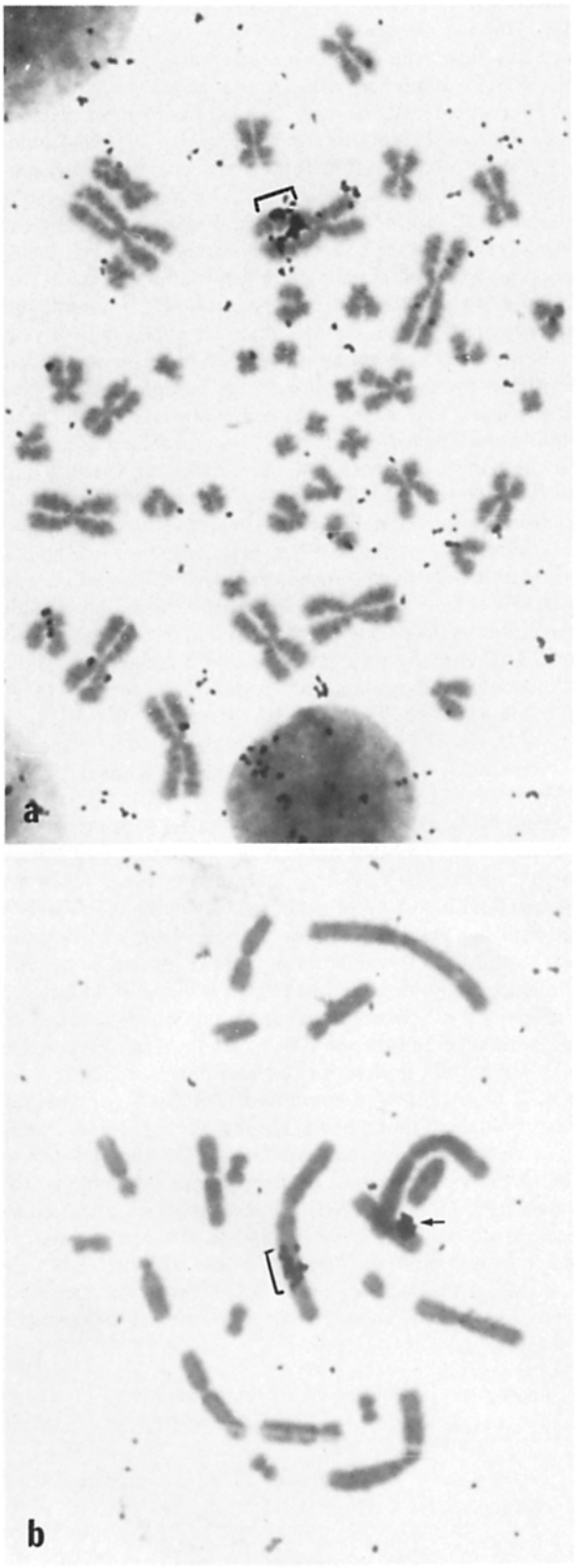
Cell line	DHFR gene equivalents‡ relative to the DC-3F line	Increase in DHFR activity relative to the DC-3F line§
DC-3F/MQ10	2.95 ± 0.02	2.9
DC-3F/MQ31	3.27 ± 0.12	3.9
DC-3F8/A55	2.15 ± 0.0	4.6

* $t½$ is the time required for 50% hybridization of the recombinant insert probe, computed according to the equation $1/f_{ss} = t/t½ + 1$ (15), where f_{ss} is the fraction single stranded and t is the time of sample incubation.

‡ Since equal amounts of genomic DNA were used in these hybridization experiments, the data do not discriminate between diploid and tetraploid nuclear DNA contents. Hence, the near-tetraploid lines DC-3F/MQ10 and DC-3F/MQ31 actually contain approximately six times more DHFR gene copies per cell than the near-diploid control line DC-3F. The value listed is the average obtained from two separate experiments.

§ Data taken from Biedler et al. (9).

The abnormally banding region in the near-diploid subline DC-3F/A55 contains three copies of the DHFR gene, the fourth copy resident on the single normal homolog. Although we have not done hybridizations *in situ* with subline DC-3F/MQ31, we assume both of the abnormally banding homologs in this near-tetraploid line to each contain five DHFR gene copies, and the two normal homologs to contain one copy each.



1 *a*. (*b*) Subline DC-3F8/A55. Bracket indicates a grain cluster on the probable chromosome 2 with the abnormally banding region. Grains are located over a region corresponding to the constricted, central region of the chromosome arm (Fig. 1 *c*) and not the proximal abnormally banding portion. Arrow indicates a probable contaminant grain cluster unique to this cell. 1750× magnification.

FIGURE 3 Hybridization of radiolabeled pDHFR6 to metaphase chromosomes. (*a*) Subline DC-3F/MQ10. Bracket delineates a cluster of silver grains over a region corresponding in size and chromosomal location to the abnormally banding region depicted in Fig.

Hybridization *in situ* of another near-tetraploid subline, DC-3F/MQ20, containing two identical abnormally banding homologs, has shown that both contain amplified DHFR genes (18).

DISCUSSION

The hybridization kinetic data presented here show that three independently derived antifolate-resistant Chinese hamster lung cell lines exhibiting low level increases in DHFR activity, i.e., three to five times that of controls, contain amplified DHFR genes. Since in each case these genes are present within abnormally banding regions of chromosome 2q, it is not possible to estimate what effect, if any, chromosomal rearrangements may have on DHFR gene expression. However, and although the formal possibility exists that not all amplified genes are transcribed, the correlation between the relative DHFR gene copy number and the relative amount of DHFR activity (Table II), coupled with other data (19, and P. W. Melera, C. A. Hession, J. P. Davide, K. W. Scotto, J. L. Biedler, M. B. Meyers, and S. Shanske, manuscript submitted for publication) which shows that the measured increases in DHFR activity in these cells is associated with actual increases in the amount of enzymes present, strongly suggest that amplification of DHFR gene number is the predominant factor in regulating the level of DHFR activity in these drug-resistant sublines. Similar results have recently been reported by Kaufman and Schimke (20) who have shown via Southern blots that Chinese hamster ovary (CHO) cells selected with low concentrations of methotrexate (MTX) and overproducing low levels of DHFR, also contain increased numbers of DHFR genes. The failure in both cases to identify cell lines containing promoter-type mutations is of genetic interest, and although the sampling size is small, may reflect the greater frequency with which amplification mutants occur vs. point mutations which affect promoter efficiency. Indeed a single point mutation may not be effective in modifying the efficiency in vivo of the apparently large promoter regions associated with eukaryotic genes (21) and would, therefore, not be detected.

The presence of DHFR genes within the abnormally banding regions of chromosome 2q equates these regions, insofar as they indicate the location of amplified DHFR genes, with the HSRs found on chromosome 2q in cells that overproduce DHFR by more than 100-fold. HSRs are regarded as uniformly packaged tandem repeats of amplified DNA. Estimates of the size of these repeats, i.e., the size of the amplification unit, have been made by dividing the amount of DNA within an HSR (estimated from chromosomal length determinations) by the total number of DHFR gene copies present in the cell. The primary assumptions are that all the amplified genes are in the HSR, that all amplification units within an HSR are the same size, and that the packing ratio of DNA within an HSR is similar to that of the normal chromosomes. Several estimates have predicted that for CHO (2) and mouse cell lines (4, 5), the amplification unit contains ~500–3,000 kilobase pair (kbp) of DNA. However, an estimate of 135 kbp has recently been reported for a CHO line containing 700–1,000 DHFR gene copies (6). Assuming the Chinese hamster lung cell genome to contain 2×10^9 bp of DNA and the diploid parental cell line DC-3F to contain two copies of the DHFR gene, we can calculate an amplification unit size of ~300 to 700 kbp for several of our sublines in which HSR length (9) and DHFR gene copy number (3) are known. If similar calculations are made for sublines containing abnormally banding regions, however, extremely large unit sizes result, DC-3F/MQ31 being the largest and containing 35,800 kbp of DNA, followed by DC-3F/A55 with 17,700 kbp of DNA, and DC-3F/MQ10 with 7,066 kbp of DNA per unit.

In addition to these bewildering estimates of size, no obvious correlation exists between the length of an abnormally banded region and the number of DHFR genes within it. For example, the abnormally banded segment of DC-3F/MQ31 is three times the size of that in subline DC-3F/MQ10 (9), yet DC-3F/MQ31 is estimated to contain five DHFR genes per abnormally banded region while DC-3F/MQ10 contains nine (see Results). A similar comparison between sublines DC-3F/MQ31 and DC-3F/MQ20 reveals that abnormally banded regions of the same size, i.e., approximately twice the length of chromosome 2p (9), can contain widely different numbers of DHFR genes, e.g., the abnormally banded region of DC-3F/MQ31 contains five DHFR genes while that in subline DC-3F/MQ20 contains 20 to 40 (3). Apparently, therefore, DHFR gene copy number and abnormally banding region size can vary independently, the magnitude of one having little or no effect on the other. Since, in all cases, the abnormally banding regions represent the locations of amplified DHFR genes, these discrepancies may conceivebly result from wide variations in the size of the DHFR gene amplification unit. Alternatively, however, and perhaps more likely considering the estimated sizes of these units, abnormally banding regions may contain, in addition to amplified DHFR genes, other amplified sequences and disrupted chromosome 2q DNA resulting from multiple insertions of varied DNA sequences along chromosome 2q in sufficient amounts to disrupt normal banding patterns. Such disruptions in banding pattern have been reported to accompany the integration of transforming DNA into host cell chromosomes (22). If DHFR gene amplification proceeds by a mechanism which includes an extrachromosomal phase through which copies of the DHFR gene (18, 23) and other DNA sequences pass, followed by integration of these sequences at various sites along chromosome 2q, then disruptions to banding patterns similar to those resulting from integration of transforming DNA could result. This would help to explain why abnormally banding regions and HSRs have been found to be located at many different sites along chromosome 2q (18).

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