Quantitative correlation of homogeneously stained regions on chromosome 10 with dihydrofolate reductase enzyme in human cells

(cytogenetic localization/methotrexate resistance)

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ABSTRACT Gene amplification may be visualized within a chromosome as a homogeneously stained region (HSR) and HSRs have rarely been reported in human tumor cells with identification of the amplified gene. A parental line and seven clones derived from KB cells resistant to methotrexate (MTX) contain dihydrofolate reductase (DHFR; tetrahydrofolate dehydrogenase; EC 1.5.1.3), ranging from 0.007 unit/mg in the parent to 0.369 unit/mg in clone 7A with a 13,000-fold increase in resistance to MTX. The enzyme is identical to DHFR from other human sources, including that from leukemic patients. A HSR localized to the long arm of chromosome 10(q26) is present in clones selected at or above 2.5 μ M MTX. Increase in number of 10q per cell, increase in number of HSR, and increasing amounts of DHFR correlate well. The chromosome change is stable with time as is enzyme production even in the absence of selection by MTX. No clone has shown double minutes. The gene copy number is low. The stability and low gene copy in the presence of large HSRs differ from the pattern described for murine tumors. A human gene for DHFR may be associated with the long arm of chromosome 10.

Gene amplification is inducible in mammalian cells by incremental exposure to cytotoxic drugs and is often accompanied by the appearance of anomalous chromosomes. Resistance to methotrexate (MTX) by overproduction of dihydrofolate reductase (DHFR; tetrahydrofolate dehydrogenase; EC 1.5.1.3) results from amplification of the DHFR gene copy number in murine cells (1). A chromosomal entity, the homogeneously stained region (HSR), has sometimes been observed in association with drug resistance (2) and shown to be the site of gene amplification (3). Another chromosomal counterpart of amplified gene function is the double minute chromosome fragment (DM), a structural alternative to the HSR within a cell population (4). The DM lacks a centromere and is unstable, whereas the HSR, integrated into a centric chromosome, replicates and segregates normally with cell division (5). Both chromosomal anomalies have been observed in human tumor cells, usually without precise identification of the gene product (2, 6).

We have induced HSR formation in a human tumor-derived cell line by exposure to increasing concentrations of MTX. We found a quantitative relationship between increased DHFR production and a series of chromosomal events which included both numerical and structural modifications. The region of gain and HSR formation suggests possible localization for a human DHFR gene (*dhfr*). The stability of drug resistance and relatively low gene copy numbers associated with HSR in these human cells differ from results in rodent cell systems (7, 8).

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MATERIALS AND METHODS

The KB line, originally derived from an oral carcinoma, now appears to be a HeLa contaminant, based upon enzymic and cytogenetic markers (9). Parental KB cells can survive exposure to 0.01 μ M MTX. Mass cultures were exposed to 0.1 μ M MTX for over 6 weeks; the survivors were selected subsequently by stepwise exposure to 3-fold increments of MTX. Clones, derived from mass culture by isolation with cloning rings, were selected at concentrations shown in Table 1; DHFR was measured as in ref. 10. Metaphase preparations, obtained 24–48 hr after subculture, were stained with aceto-orcein for DMs and by standard methods for G, Q, and C banding.

Probes were prepared from pDHF 26 plasmid DNA (kindly provided by R. T. Shimke), restricted with *Pst* I to obtain the DHFR cDNA insert. This was then restricted with *Bgl* II to obtain the 675-base pair DNA, a fragment corresponding to the coding region of the mRNA (11). These fragments were separated on agarose and polyacrylamide gels; isolation of restriction fragments was performed by electroelution onto dialysis membranes for agarose gels (12) and then by excision and elution (13) for polyacrylamide gels.

For estimation of DNA copy numbers, high molecular weight DNA was extracted from the cells by protease K treatment and phenol/chloroform extraction (14). Known amounts (at least three different amounts for each DNA, ranging from 0.2 to 4 μ g) were heat-denatured and spotted onto nitrocellulose paper (15). Hybridization with the 32 P-labeled probe was performed at 65°C for 16 hr in 0.9 M NaCl/3 mM NaH₂PO₄, pH 7.0/0.3 mM EDTA/0.5% NaDodSO₄ with a 20-fold excess of bacteriophage T4 DNA added (16). After autoradiography, squares of nitrocellulose corresponding to the spots were assayed for radioactivity in a Beckman LS100 C scintillation counter. Hybridizations were done with 32 P-labeled, nick-translated DNA from the JW/02 human β -globulin cDNA clone for accurate estimation of DNA concentration and these values were used as controls. Copy number values are reported on the basis of 1 for KB parental DNA.

RESULTS AND DISCUSSION

Clones growing in increased concentrations of MTX produce increased amounts of DHFR (Table 1). The DHFR isolated from clone 6B is identical to enzyme from the parental KB line and to enzyme freshly isolated from human leukemia (acute myelocytic) cells. Its M_r is 20,000, it forms a single band in

Abbreviations: MTX, methotrexate; DHFR, dihydrofolate reductase; *dhfr*, dihydrofolate reductase gene; HSR, homogeneously stained region in chromosome; DM, double minute chromosome fragment; q, longer arm of a bi-armed chromosome.

Table 1. Properties of MTX-resistant KB sublines

Cell line	MTX		DHFR, unit/mg [†]	Chromosomes*		
	Conc., μ M	Relative resistance		Modal chromosome no. (range)	HSR	10q [‡]
Parent	—	1	0.007	78 (59–78)	None	3
2D	0.25	10	0.066	71 (51–72)	None	3–4
4A	2.5	600	0.160	68 (58–71)	1	3
6A	25	6,500	0.331	70 (60–72)	2	4–5
6B	25		0.284	69 (66–70)	2	4–5
6C	25		0.309	69 (66–73)	2	4
6D	25		0.279	70 (61–73)	2	4
7A	75	13,000	0.369	71 (59–72)	2–4	4–6

* Twenty to forty (or more) cells analyzed per clone.

[†] Measured after 2 weeks or more in MTX-depleted medium.

[‡] In all but clone 2D (see text), these values represent the intact chromosome 10 per cell.

NaDodSO₄ gel, and the specific activity after purification is 60 units/mg of protein (10). Each clone, judged by NaDodSO₄ electrophoresis, shows no apparent differences in protein profile except for DHFR. Kinetic properties of enzyme from the three sources are identical in substrate utilization, MTX binding, and isoelectric focusing (at pH 7.3). In these respects this DHFR differs from murine DHFR derived from the S 180 line (10). Therefore, the resistant clones of KB appear to produce increased amounts of unaltered human DHFR.

The uncloned parental karyotype is near triploid with several well-known HeLa marker chromosomes (17). Many chromo-

somal rearrangements have resulted from breakage and recombination at or near centromeres. The least resistant clone (2D) did not contain a HSR. The localization of a HSR in the long arm (q) of chromosome 10 at higher levels of MTX resistance suggested analysis for numerical increases in this region in clone 2D. Karyotypic analysis of 20 parental cells revealed a pair of chromosome 10 in 3 cells and trisomy for chromosome 10 in 17 cells. No recombinants involving the 10q were identified in the parent or any clone other than 2D. Despite a lower modal chromosome number than the parent line (Table 1), clone 2D showed an absolute increase in copies of 10q. Intact chromosome 10 were present in one to four copies per cell; in addition, in each cell there were one or two copies of a recombinant in which the p arms resembled an 11q and the q arms were identifiable as 10q. The average number of 10q copies in 24 cells from clone 2D was 3.6, in contrast to an average below 2.9 for the parent. A 10-fold increase in MTX resistance resulted in the appearance in cells of clone 4A of a single, otherwise normal, chromosome 10 with a HSR in the region 10q26. All clones isolated in 25 μ M MTX contained a large metacentric marker with two HSRs (Fig. 1). The arms are mirror images of one another, suggesting that the marker arose by isochromosome formation (misdivision at the centromere) (18). C banding demonstrated two small centromeric masses, each similar to that of a normal chromosome 10 from the same cell. At the highest concentration studied (clone 7A), an additional HSR-bearing chromosome was found. At this exposure the generation time was prolonged and increased numbers of chromosome breaks were observed.

Because there is intercellular variation in chromosomal con-

FIG. 1. Karyotype from clone 6B grown in 25 μ M MTX. The large marker is shown next to a pair of normal chromosome 10. The proximal portion of each arm resembles 10q in band position and stain intensity. Two HSRs (arrows), characteristically pale staining with G or Q banding, are located distally at positions 10q26. A faint repeated banding is evident within the HSRs. Several well-known HeLa markers and other recombinant chromosomes are shown in the bottom line.

traction, an intracellular reference was developed to obtain relative measures of the amount of HSR in each clone. A ratio was obtained by dividing the cumulative length of the HSR regions of an individual cell by the average length of the 1q arms from the same cell. The length of the HSR remained constant, whereas the numbers of HSR increased (Table 2) and the amount of enzyme was proportional to the relative length of HSR per clone (see Tables 1 and 2).

Clone 6B has been cultured for over 14 months in MTX-free medium. In the presence of MTX, DHFR is found in both bound and free forms. With removal of cells into MTX-free medium the enzyme level stabilizes at approximately 1/4 of the original value within 2 weeks; the higher value is restored within 3 days of reexposure to MTX (19). DMs are rare in these cultures; only one cell with a DM was found in 100 metaphases of clone 6B 8 months after MTX depletion. The HSR-bearing chromosome was present in every cell and its size was unchanged (Table 2). The level of DHFR was identical to that found within 2 weeks after depletion. Thus, the amplified gene appears to be stable with time, both in function and with respect to chromosomal integration.

Hybridizations *in situ* by methods used in identification of mouse *dhfr* genes (20) were unsuccessful. The same mouse probe that codes for mouse DHFR with over 90% homology to the human DHFR (21) was used to estimate gene copy number. DNA extracts of clone 6B showed a 9-fold increase over the parent in ability to hybridize with the mouse probe on nitrocellulose. Thus, less than five gene copies are estimated to correspond to each HSR. Corroborative data on other clones have been obtained (22).

Chromosomal events provided means for increase in a specific subchromosomal region coincident with increasing drug resistance. The apparent sequence in these experiments began with increase in the number of chromosomes (no. 10) or partial chromosomes (10q) presumed to bear relevant information. As drug resistance increased, a HSR appeared (10q26), followed by formation of an isochromosome, doubling the HSR, and finally, selection for additional HSR-bearing chromosomes.

The assignment of a human *dhfr* to the HSR locus is tentative for several reasons. Localization of amplified *dhfr* genes in the hamster is not entirely uniform (8). It has been suggested that

gene amplification occurs in chromosomal fragments followed by reintegration, not necessarily at the native gene location (23). An MTX-resistant human leukemia line is marked by HSRs on two different (no. 6 and no. 19) chromosomes (although its enzymic properties are not fully defined) (24). Furthermore, the human *dhfr* family is complex and probably includes four loci (25).

In other species massive increases in gene copy (50- to 200-fold) accompany HSR formation (26). The amplified murine unit is known to be much larger in size than the *dhfr* (20, 27), but the discrepancy between the number of gene copies and the amount of HSR is much greater in this human system than has been described previously.

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Table 2. Relative HSR lengths

Clone	MTX*	HSR, no.	HSR/1q	
			Ratio	Range
4A	+	1	1.01	(0.90–1.17)
6A	+	2	2.06	(1.64–2.23)
6C	+	2	2.33	(2.20–2.49)
6D	+	2	2.25	(2.61–2.55)
7A	+	2–4	3.23	(2.17–4.14)
6B	+	2	2.40	(2.30–2.56)
6B†	–	2	2.57	(2.36–3.24)
6B‡	–	2	2.64	(2.11–3.08)§
6B¶	–	2	2.61	(2.48–2.75)

Relative HSR length measurements were based on 5–20 cells per clone.

* MTX-containing (+) and MTX-free (–) culture medium.

† Three weeks in MTX-free medium.

‡ Eight weeks in MTX-free medium.

§ One cell, with an additional (tandem repeat) HSR, showed a ratio of 4.27.

¶ Eight months in MTX-free medium.

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