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Thymidylate Synthase-Deficient Chinese Hamster Cells: A Selection System for Human Chromosome 18 and Experimental System for the Study of Thymidylate Synthase Regulation and Fragile X Expression

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SUMMARY

Chinese hamster lung (CHL) V79 cells already deficient in hypoxanthine phosphoribosyltransferase were exposed to uv light and selected for mutations causing deficiency of thymidylate synthase (TS) by their resistance to aminopterin in the presence of thymidine and limiting amounts of methyl tetrahydrofolate. Three of seven colonies chosen for initial study were shown to be thymidylate synthase deficient (TS^-) by enzyme assay, thymidine auxotrophy, and their inability to incorporate labeled deoxyuridine into their DNA in vivo. Complementation analysis of human × TS^- hamster hybrids revealed that TS activity segregated with human chromosome 18. Southern analysis of a panel of 14 human × hamster hybrids probed with complementary DNA from mouse TS confirmed the chromosome assignment of TS to human chromosome 18; quantitative Southern blotting using unbalanced human cell lines further localized the gene to 18q21.31→qter. Another hybrid was generated that contained a human X chromosome with the Xq28 folate-dependent fragile site as its only human chromosome in a hamster TS^- background. The fragile site could be easily and reproducibly expressed in this hybrid without the use of antimetabolites simply by removing exogenous thymidine from the medium. These TS-deficient cells are useful for: (1) somatic

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cell genetics as a unique selectable marker for human chromosome 18, (2) studies on regulation of the TS gene, and (3) analysis of the fragile (X) chromosome and other folate-dependent fragile sites.

INTRODUCTION

Thymidylate synthase (TS) (E.C. 2.1.1.45) is an essential enzyme in the biosynthesis of the DNA precursor thymidylate triphosphate [1]. It has been suggested that the enzyme is part of a multienzyme complex, the replitase, which contains a number of enzymes important for DNA synthesis [2]. TS activity varies throughout the cell cycle, rising dramatically prior to S phase [3, 4]. Because of this apparent cell-cycle control and its crucial role in DNA synthesis, TS is the subject of expanding somatic cell and molecular genetic research. Mouse cell lines have been isolated that either overproduce [5] or lack TS [6]. The overproducing cell line was used as a source of mRNA for the successful molecular cloning of complementary DNA (cDNA) for mouse TS [7]. TS-deficient cells have been used for DNA-mediated gene transfer of the human TS gene and isolation of human genomic fragments [8].

As a first step in a study of the control of the human TS gene, we have isolated Chinese hamster cells that lack TS and used these cells to map provisionally the TS gene to human chromosome 18 by analyzing a panel of somatic cell hybrids selected for complementation of thymidine (Td) auxotrophy [9]. This chromosomal assignment was confirmed and refined by Southern analysis using mouse TS cDNA as probe against DNA from another panel of independently derived hamster/human hybrids and human aneuploid cells. A TS-related sequence, possibly a pseudogene, was also found in Southern blots of human DNA probed with TS and shown not to be syntenic with TS using the same hybrid panel.

TS has also become a focus of interest for cytogeneticists studying folate-dependent chromosomal fragile sites [10]. Fragile sites are regions of apparently incompletely condensed chromatin that can be induced at specific chromosomal locations in the cells of some individuals and appear as achromatic gaps in metaphase chromosomes. One class of fragile sites, the folate-dependent sites, are induced by exposing cells during S phase to 5′-fluorodeoxyuridine (FUdR) [11], whose metabolite 5-fluorodeoxyuridine monophosphate directly inhibits TS, or by depriving cells of tetrahydrofolate, a cofactor for TS, using folate-deficient medium [12] or methotrexate [13]. It has been assumed that a deficiency in Td metabolites for DNA synthesis is the final common pathway by which these different methods of inducing expression all act. We have used the TS-deficient cells described here to form hybrids with cells from a male patient affected with X-linked mental retardation and the folate-dependent Xq28 fragile site [14] as part of our ongoing study of the expression of this fragile site in somatic cell hybrids [15]. With such TS-

deficient hybrids, we have directly tested and confirmed that thymidylate deprivation per se is indeed responsible for expression of this fragile site.

MATERIALS AND METHODS

Cells and Cell Culture

RJK 88 is a Chinese hamster lung V79 line that is deficient in hypoxanthine phosphoribosyltransferase due to gene deletion [16]. GM4025 is a human lymphoblastoid cell line from a male with fragile X syndrome obtained from the Human Genetic Mutant Cell Repository (Institute for Medical Research, Camden, N.J.). This cell line expresses the fragile site in 30%–40% of mitotic cells following FUdR treatment. KD is a mosaic human lymphoblastoid cell line showing a 46,XX,del(18q21.31:) karyotype in 90% of cells and 46,XX in 10%. DR is a human lymphoblastoid cell line from a patient with a 47,XX,+iso(18p) karyotype. JB is a normal human 46,XX cell line. Unless otherwise specified, cells were grown in Dulbecco's modified Eagle medium with high glucose (DMEM) supplemented with 10% heat-inactivated fetal calf serum (CFCS) in 10% CO_2 atmosphere. Dialyzed fetal calf serum (DFCS) was obtained by exhaustive dialysis of CFCS against phosphate-buffered saline at 4°C followed by filter sterilization.

Isolation of TS-deficient Cells

RJK88 was plated in ten 100-mm dishes at 10^6 cells per dish, allowed to attach overnight, and then exposed to uv light from a germicidal lamp at a dose previously determined to produce 10% survival. The cells were allowed to recover for 3 days in DMEM + CFCS + 10^{-5} M Td and then exposed to selection medium, consisting of DMEM and DFCS and 2×10^{-8} M folinic acid, 10^{-6} M aminopterin, 10^{-5} M Td, and 1.5×10^{-7} M cyanocobalamin. This selection medium (FAT medium) is a modification of the original formulation of Ayusawa et al. [6] and was developed by adopting the levels of aminopterin and Td used in HAT medium [15] and then testing various concentrations of folinic acid to find the level at which no survivors were found among 10^8 RJK88 cells exposed to the FAT medium without prior mutagenesis. Cells that did survive FAT selection after uv mutagenesis were isolated using cloning cylinders, transferred to T25 flasks in 2×10^{-8} M folinic acid and 10^{-5} Td, and then maintained in DMEM and CFCS with 10^{-5} M Td. The TS-deficient phenotype of FAT-resistant cells was assessed in three ways. First, cells were set out at varying densities in duplicate plates with DMEM and DFCS in the presence or absence of 10^{-5} M Td. Surviving colonies after 18 days were stained with methylene blue in methanol and counted. Second, cells were placed in DMEM and DFCS for 3 hrs and then exposed to either 10^{-8} M [^{3}H]Td or 10^{-8} M [^{3}H]-6′-deoxyuridine (10 Ci/mmol) either in the presence or absence of 10^{-5} M unlabeled Td. The cells were then lysed in situ and their DNA acid-precipitated on glass-fiber filters and counted in Liquifluour scintillation fluid. Corrected incorporation was defined as the counts per minute (cpm) in precipitated DNA when unlabeled Td was not present during the labeling period minus the cpm in precipitated DNA when 10^{-5} M unlabeled Td (a 1,000-fold excess) was present during labeling. Relative incorporation (R.I.) was calculated as the ratio of the corrected incorporation for a TS-deficient line to that of wild-type RJK88. Finally, TS activity was assayed directly. Growing cells from plates at less than 50% confluence were harvested, cell extracts were made by lysing cells in 10 mM Tris, pH 7.0, 25% w/v Sucrose, and 0.5% NP-40, and both TS activity and protein concentration were measured. The TS was measured by a published method that relies upon measuring the release of [^{3}H]H_2O from [5-^{3}H]dUMP [3]. Protein was measured by the method of Lowry et al. [17] using bovine serum albumin as standard. Specific TS activity is expressed as pmol TMP/min-mg.

Somatic Cell Hybridization

A TS-deficient cell line and GM4025 were fused at a ratio of 1:5 in suspension using 50% (w/v) polyethylene glycol 1,000 as described [18]. Two days following hybridization, the medium was removed and replaced with either DMEM and DFCS, without thymidine, or with DMEM and CFCS with hypoxanthine (10^{-4} M), azaserine (5×10^{-5} M), and Td (10^{-5} M) (HZT) [15]. Surviving colonies were allowed to grow to the 300–400 cell stage and then transferred using cloning cylinders to separate T25 flasks. Individual colonies were grown and analyzed cytogenetically by trypsin G-banding and G-11 staining [19] and biochemically by TS assay.

Southern Analysis

High molecular weight DNA was isolated as described from human lymphocytes and cultured cells [18]. Ten micrograms of DNA were digested with 3–4 U/μg of restriction endonuclease, subjected to electrophoresis in Tris-acetate buffer, pH 8, at 40 V overnight in 0.8% agarose, and then transferred to nitrocellulose by published procedures [20]. Nitrocellulose filters were prehybridized and hybridized as described except that only 30% formamide was used [17]. The probe used in all cases was a 750-base pair (bp) internal *Pst*I-*Pst*I fragment from cloned complementary DNA (cDNA) for mouse TS (pMTS-3) obtained from L. F. Johnson [7]. The fragment was isolated from low-melting agarose and nick-translated to $> 10^8$ cpm/μg [21].

Chromosomal assignments for human TS and related genes were made by determining the concordance between chromosomal content of hybrids and human-specific restriction fragments on Southern blot. Densitometry tracings of the normal and unbalanced cell line DNAs were performed on lanes with equal DNA loading by matching the absorption at the 2.4-kilobase (kb) human *Eco*RI fragment determined to be nonsyntenic with the functional TS gene (vide infra).

Fragile Site Expression

Somatic cell hybrids were plated at a density of 2.5×10^5 cells per T25 flask in HT medium (DMEM + hypoxanthine + Td). The following day, the medium was removed and replaced with experimental medium consisting of DMEM with either 5% or 10% CFCS and varying concentrations of Td. Colcemid (10 μg/ml) was added 4, 6, and 8 hrs later and the cells harvested for analysis of fragile site expression [15].

In another series of experiments, fragile site expression was determined in somatic cell hybrid cells following release from Td starvation. Cells were exposed for 15 hrs to DMEM and 10% CFCS without thymidine. Following the 15-hr period of Td starvation, the medium was replaced with fresh DMEM containing 5% CFCS and either 0 or 10^{-5} M thymidine. Cells were harvested 6 or 8 hrs later for cytogenetic analysis [15].

RESULTS

Isolation of TS-deficient Cell Lines

Following uv mutagenesis and FAT selection, 1–2 colonies per plate were found to survive and grow. No colonies were seen in plates not exposed to the mutagen. Only one such colony from each plate was isolated for study to insure the independence of the isolates. Because a number of mechanisms in addition to loss of TS may cause FAT resistance (FAT^r) (aminopterin resistance among them), the TS activity of the surviving colonies was assayed indirectly in vivo and directly in vitro. Of seven FAT^r selected for study initially, three (88.12, 88.13, and 88.15) failed to form viable colonies in the absence of exogenous Td.

TABLE 1

TS ACTIVITY IN CHINESE HAMSTER CELLS AND MUTANTS

CELL LINE	[^{3}H]THYMIDINE INCORPORATION* (cpm × 10^{-3})			[^{3}H]DEOXYURIDINE INCORPORATION* (cpm × 10^{-3})			TS SPECIFIC ACTIVITY (pmol/min-mg)
	−T	+T	RI	−T	+T	RI	
88	58	3	...	62	1	...	64.7
88.12	55	2	0.96	6	2	0.07	< 3
88.13	52	2	0.90	1	1	0	< 3
88.15	55	3	0.95	2	2	0	< 3

* Incorporation of isotope in the absence (−T) and presence (+T) of excess (10^{-5} M) unlabeled Td.

Addition of 10^{-5} M Td restored plating efficiency, although not to the levels of parental RJK88 cells, in that cells demonstrated poor survival when at low density. A more specific assessment of TS activity in vivo was made by measuring incorporation of [6-^{3}H]deoxyuridine into cellular DNA. This tritiated deoxynucleoside is phosphorylated to [6-^{3}H]dUMP, which is converted by TS into [6-^{3}H]dTMP and subsequently incorporated into DNA. As shown in table 1, 88.13 and 88.15 incorporated into their cellular DNA less than 1% of the radioactivity incorporated by control wild-type cells. Clone 88.12 had some residual TS activity in vivo, incorporating 6% of normal levels of [^{3}H]deoxyuridine. In vitro, all three cell lines had TS activity that was unmeasurable, below the lower limit of detection in this assay of 3 pmol/min-mg, or 5% of wild-type activity.

One clone, 88.13, was chosen for somatic cell hybridization studies. Reversion without prior mutagenesis was assessed by plating 2×10^6 cells in DMEM and DFCS without Td: no viable colonies were found after 3 weeks. The plating efficiency of 88.13 in thymidine is very dependent on cell density, with efficiency increasing significantly with initial cell density. Thus, it is very difficult to determine true plating efficiency at high density since it is much greater than would be calculated from colony counts at low plating densities. With this limitation in mind, plating efficiency at high density could be estimated to be in the 10%–50% range, which provides an estimate of spontaneous reversion frequency of $0.5–2.5 \times 10^{-7}$. Td auxotrophy was phenotypically stable during 2 months of continuous culture in Td without FAT selection pressure. Growth at 30°C did not relieve 88.13 of its Td auxotrophy, making it unlikely that a temperature-sensitive mutation was present. Southern analysis of 88.13 DNA cut with *Eco*RI or *Pst* and probed with mouse TS cDNA did not reveal any differences with the RJK 88 parent, ruling out a large gene deletion in 88.13 (data not shown).

Somatic Cell Hybridization

From an initial fusion of 10^7 88.13 cells and 5×10^7 GM4025 lymphoblasts, 14 colonies survived in DMEM and DFCS without Td. Of these, 10 could be isolated and grown to bulk culture for analysis. All were found to have regained

TABLE 2

CHROMOSOME CONSTITUTION OF HYBRIDS SELECTED FOR Td PROTOTROPHY

Hybrid	Chromosome 18	Chromosome 22	Cytogenetically unidentifiable G-11+ fragment	Other human chromosomes
1	+	−	+	9
2	−	+	+	None
3	+	+	−	5, 14
4	+	+	−	Many
7	+	+	−	Many
8	+	+	−	X, 1, 2, 9, 12, 13, 21
10	−	+	+	None
14	+	+	−	Many
3-FAT^r	−	+	−	5, 14
8-FAT^r	−	+	−	X, 12, 13, 21

TS activity. In the eight hybrid lines whose chromosomes could be identified cytogenetically, acquisition of TS activity was correlated with the presence of chromosome 18 in six and with chromosome 22 in seven of the lines (table 2). All other chromosomes were discordant with TS activity in four or more of the eight hybrids. In addition, in all three hybrids that were discordant for either chromosome 18 or 22, G-11 pale-staining material, characteristic of human chromatin, was seen but could not be identified as to chromosome of origin by G-banding. Thus, the data pointed to chromosome 18 or 22 as the location for TS but were insufficient to decide between them. However, hybrids 3 and 8 contained cytogenetically normal chromosomes 18 and 22 and provided an opportunity to determine whether 18 or 22 carried the TS gene by back selecting against TS and observing which chromosome was lost. Both hybrids, when back selected in FAT, were found to have retained chromosome 22 but to have lost chromosome 18 in 20 of 20 mitoses scored. TS activity was again absent in the back-selected hybrids. Therefore, although the number of hybrids was small, the original concordance with 18 and 22 and the specific loss of 18 in two back-selected FAT^r derivatives allowed a provisional assignment of the human TS gene to chromosome 18.

Mapping of the TS Structural Locus by Southern Analysis

We sought further evidence for the assignment of the TS structural gene to chromosome 18 by Southern analysis of a panel of hybrids that were independent of those derived by complementation of the Td auxotrophy in 88.13. By probing Southern blots of DNA from a panel of hamster/human hybrids with mouse TS cDNA, the chromosomal location of the TS structural gene as well as any cross-hybridizing-related sequences could be determined. Such a panel was already in existence and had been used to map the pseudogenes of argininosuccinic acid synthetase [22] and hypoxanthine phosphoribosyltransferase [23]. DNA from these hybrids was digested with the restriction endonuclease *Eco*RI and probed with the 750 bp *Pst*I-*Pst*I- internal fragment of mouse TS cDNA. *Eco*RI was chosen because preliminary experiments using hamster

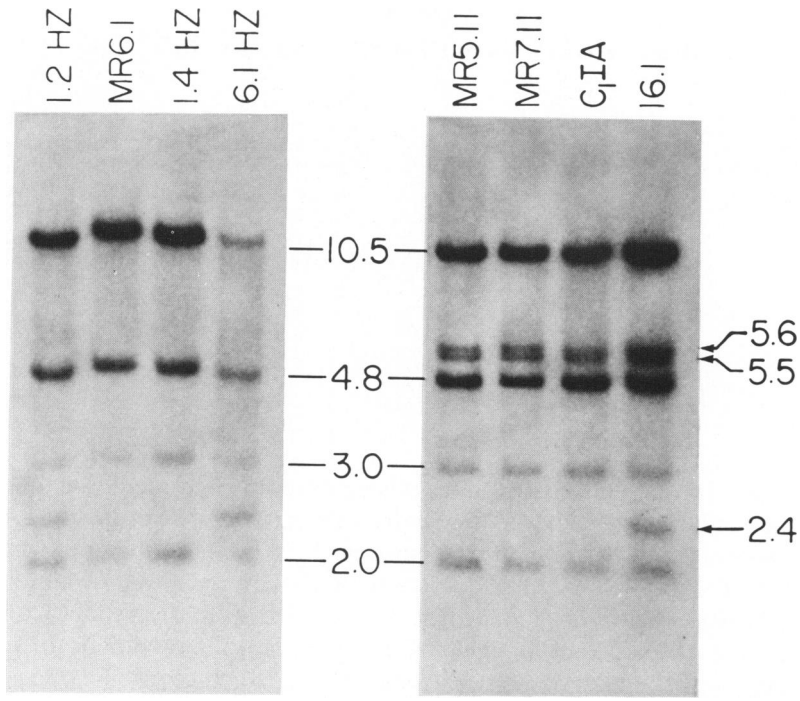

FIG. 1.—Southern blot analysis of *Eco*RI-cut DNA from eight of the hybrids listed in table 3. The sizes of the hamster parental fragments are shown *in the center*. The human specific fragment sizes are shown *to the right*.

and human DNA cut with a variety of enzymes had shown human *Eco*RI fragments of 6.8 kb, 5.6 kb, 5.5 kb, and 2.4 kb that were readily distinguishable from the hamster *Eco*RI fragments of 10.5 kb, 4.8 kb, 3.0 kb, and 2.0 kb. The 6.8-kb, 5.6-kb, and 5.5-kb *Eco*RI fragments were thought to contain the exons of the functional human gene because these fragments, but not the 2.4-kb fragment, were seen in all eight hamster-human hybrids in table 2 selected for complementation of the Td auxotrophy in 88.13 (data not shown). Furthermore, Takeishi et al. [8] reported human *Eco*RI fragments of these sizes in DNA from cells transfected to Td auxotrophy with total human DNA and probed with a human repeat sequence. A representative blot containing eight of the hybrids is shown in figure 1. Human fragments of 5.6 kb and 5.5 kb were present or absent together in any one hybrid whereas the 2.4-kb human fragment appeared independent of the others. The 6.8-kb fragment was very faint in all Southern blots of human DNA and was not well seen in this blot. As shown in table 3, the two human *Eco*RI fragments of 5.5 and 5.6 kb showed perfect concordance with chromosome 18. The 2.4-kb fragment was discordant with 18 and showed the best concordance with chromosome 19, although low discordance was also seen with chromosome 4.

With the human TS gene localized to chromosome 18, we sought to map the gene regionally by measuring the intensity of the signal of the 5.5- and 5.6-kb

TABLE 3

SEGREGATION OF *Eco*RI FRAGMENTS FROM THE TS GENE AND A RELATED SEQUENCE IN SOMATIC CELL HYBRIDS

	*Eco*RI FRAGMENTS		HUMAN CHROMOSOMES*																							
HYBRID	5.5/5.6	2.4	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
1.2HZ	−	+	+	−	+	+	+	+	−	+	+	−	+	−	−	−	−	−	−	−	+	+	+	−	+	−
MR6.1	−	−	−	+	−	−	−	−	+	−	+	−	+	−	+	+	+	−	−	−	−	−	−	−	+	−
1.4HZ	−	+	−	−	+	+	−	+	−	−	−	−	−	−	−	−	−	+	−	−	+	−	+	−	+	−
6.1HZ	−	+	+	−	+	+	−	+	−	+	−	+	+	+	+	+	−	−	−	−	+	+	+	+	+	+
MR5.11	+	−	+	+	+	−	−	−	+	−	+	+	+	−	+	−	−	+	−	+	−	−	+	+	+	−
MR7.11	+	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	+	−	−	−	+	+	−
C1Ia	+	−	−	−	+	+	+	+	−	−	−	−	+	+	−	+	+	+	+	+	−	−	+	+	+	−
16.1	+	+	−	+	+	+	+	+	−	−	−	−	+	−	−	−	−	+	−	+	+	−	+	−	+	+
1.15HZ	+	−	−	−	−	−	−	+	−	+	−	−	+	−	−	−	−	−	−	+	−	−	−	−	+	−
MR2.2	−	−	−	−	−	−	−	+	+	−	−	+	+	−	+	−	−	−	−	−	−	+	−	+	+	−
MR4.1	+	+	−	−	−	+	−	−	−	−	−	+	+	−	−	+	+	−	+	+	+	+	−	+	+	−
13.1HZ	−	−	−	−	+	−	−	+	−	−	−	−	+	−	−	−	−	+	−	−	−	+	−	+	+	−
MR6.2	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	+	−	+	+	−
2.13HZ	+	−	+	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	+	−	−	+	−	+	−
Discordant(%)		5.5/5.6	50	43	57	50	43	64	57	57	57	50	36	57	64	43	43	43	36	0	57	78	43	50	50	50
		2.4	36	43	28	7	28	36	57	28	43	36	64	43	64	43	43	43	36	57	0	36	28	64	64	21

* Human chromosome content of the cell hybrids was determined simultaneously with preparation of hybrid cell DNA. A cell hybrid was considered positive for a chromosome if ≥ 25% of the cells scored had a copy.

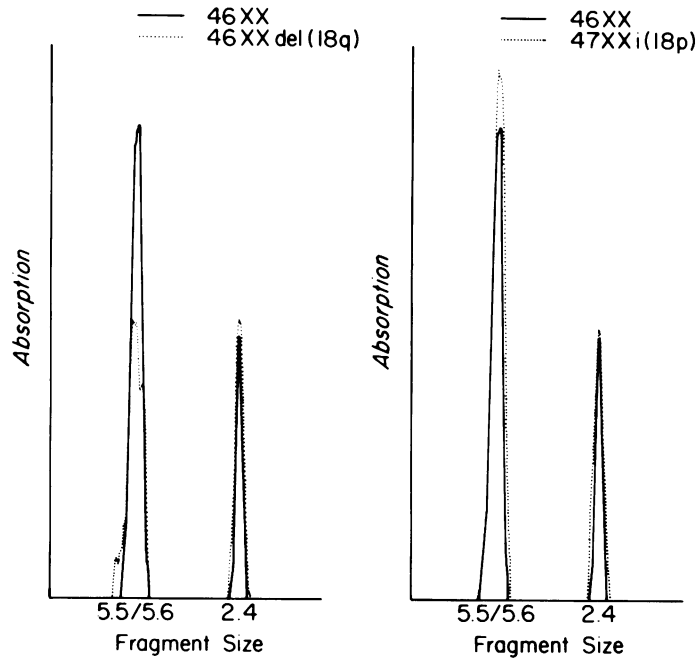

FIG. 2.—Densitometry tracings of Southern blots of DNA from a normal 46,XX female compared with a 46,XX, del(18q21.31:) cell line (*left-hand panel*) and a 47,XX, iso(18p) cell line (*right-hand panel*). DNA lanes used for densitometry were matched for total DNA loading.

*Eco*RI fragments on Southern blots of carefully matched DNA samples from KD, JB, and DR. JB is disomic for all of chromosome 18, DR is tetrasomic for 18p and disomic for 18q, and KD is monosomic for 18q21.31→qter (90% of cells) and disomic for the rest of 18. Since all three lines had normal dosage for all chromosomes other than 18, the 2.4-kb *Eco*RI fragment that is nonsyntenic with human TS served as an internal control and allowed us to choose lanes for scanning that gave identical densitometry tracings for the 2.4-kb *Eco*RI fragment. The densitometry tracings for the TS genomic *Eco*RI doublet fragments of 5.5 and 5.6 kb are shown in figure 2. The normal diploid cell line JB and DR had similar tracings, whereas KD had a substantially smaller peak, approximately half normal, indicating that the gene is localized in the segment 18q21.31→qter.

Fragile X Expression Studies

Somatic cell hybrids formed between 88.13 and GM4025 and selected for the human X chromosome using HZT medium were analyzed cytogenetically. One such hybrid, PHL3, was chosen for study because it contained a single X chromosome as its only human chromosome and was confirmed by TS assay to be TS-deficient. A series of experiments were performed to determine the optimum conditions for expression of the Xq27-28 fragile site in PHL3. As shown in table 4, experiment 1, fragile site expression was seen in mitotic

TABLE 4

FRAGILE X EXPRESSION IN HYBRID PHL3 FOLLOWING Td DEPRIVATION

Experiment	FCS (%)	Td (M)	Time (hr)	Fragile site expression rate (%)
1	10	0	4	0/20 (0%)
	10	0	6	3/20 (15%)
	10	0	8	3/20 (15%)
2	10	0	8	1/26 (4%)
	5	0	8	7/26 (26%)
	10	10^{-8}	8	0/20 (0%)
	5	10^{-8}	8	2/21 (10%)
3 (15 hrs Td starvation)	5	0	6	13/50 (26%)
	5	0	8	18/50 (36%)
	5	10^{-5}	6	0/20 (0%)
	5	10^{-5}	8	0/20 (0%)

PHL3 cells within 6 hrs of removing exogenous Td from the medium, except for that in the fetal calf serum. A representative G-banded chromosome spread is shown in figure 3. As seen in experiment 2, reducing the fetal calf serum to 5% enhanced expression while adding back low levels of Td reduced or abolished it. We inferred from these results that the amount of Td provided by 5% serum must be near the critical concentrations needed to allow cells to traverse S phase and reach mitosis yet still express the fragile site. Even higher rates of expression could be seen if the cells were starved for Td for 15 hrs prior to refeeding with 5% FCS. In experiment 3, when cells were exposed to DMEM and 10% FCS without additional Td for 15 hrs, fragile site expression rates of 26%–36% were seen 6–8 hrs after refeeding cells with 5% FCS without Td. If, however, these same Td-deprived cells were fed 10^{-5} M Td, no fragile site expression was seen 6–8 hrs following release from Td starvation. We concluded from these experiments that Td deprivation per se was a necessary and sufficient condition for expression of the fragile site on the X chromosome from GM4025 and that the levels of expression correlated with the degree of Td starvation induced in the cells. Replacement of Td in the medium completely abolished fragile site expression.

DISCUSSION

We have isolated TS-deficient Chinese hamster V79 cells and have used them in somatic cell hybridization studies to map the human TS gene to chromosome 18. The TS locus constitutes, therefore, a selectable marker for human 18, a chromosome for which no specific selection was previously available. Since both forward and back selection, using Td-less and FAT media, are available, the TS locus joins such well-known and widely used selectable loci as the hypoxanthine (HPRT) and adenine phosphoribosyltransferases and thymidine kinase as useful markers for studies in somatic cell hybridization and mutation.

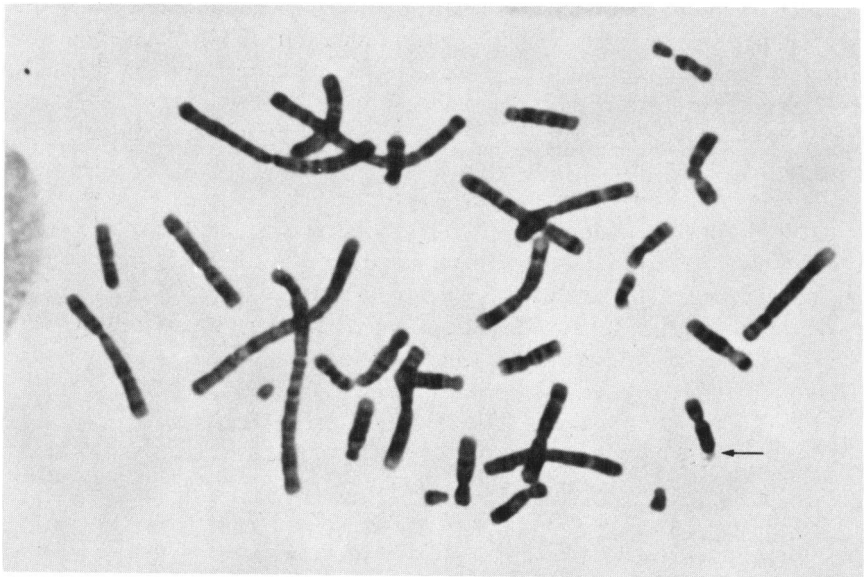

FIG. 3.—G-banded metaphase cell of cell line PHL3 showing expression of the fragile (X) against a typical V79 hamster chromosome background in cells deprived of Td.

The cell lines reported here are particularly useful since they carry two selectable markers, $HPRT^-$ and TS^-, and may therefore be used to select for two different human chromosomes.

The reason for the high rate of retention of human chromosome 22 seen in the hybrids selected for complementation of the hamster TS^- phenotype is not known. In over 50 hamster-human somatic cell hybrids we have made and analyzed using RJK88 and HPRT selection for the human X chromosome, preferential retention of chromosome 22 was not seen (unpublished data). Human chromosome 22 may be providing some growth advantage to hamster TS^- cells, perhaps because the Td prototrophy conferred by human chromosome 18 is in some way incomplete or imperfect. It is of interest that mouse TS^- cells rendered TS^+ by human DNA-mediated gene transfer showed abnormal TS regulation and seemed to require higher than normal levels of enzyme activity to sustain growth [22]. In the hybrids in table 2, there was marked variation in TS activity but no consistent elevation or depression of TS specific activity was seen (data not shown).

Assignment of TS to human chromosome 18 was confirmed using Southern blotting of hamster-human hybrids with mouse TS cDNA as probe. In addition, the gene was regionally localized to 18q21.31→qter by comparing the dosage of Southern blot signals in unbalanced human cell lines differing in the amount of human 18p and 18q they contained. Southern analysis also revealed the existence of a human sequence carried on a 2.4-kb *Eco*RI fragment that hybridized to the TS cDNA but was not syntenic with the actual gene locus. It is of interest that in a human cell line resistant to FUdR due to TS gene amplification a 2.4-

kb *Eco*RI fragment has been observed that was not amplified along with the functional gene (F. Berger, personal communication, 1984). Pseudogenes for a number of loci are well known [23–25]; some are closely linked to the functional gene, others are widely dispersed. Whether the 2.4-kb *Eco*RI fragment represents a true pseudogene or a functional cross-hybridizing sequence is not known and will require further analysis.

TS is one of a number of enzymes synthesizing DNA precursors that are thought to be present along with DNA polymerase in a multienzyme complex known as the replitase [2]. It has been suggested that the complex is a mechanism for channeling substrates for DNA synthesis from the enzymes responsible for making these substrates, such as TS, ribonucleotide reductase, and TMP kinase, to DNA polymerase [26–28]. The in vivo activity of many replitase enzymes, TS included, rises dramatically at the onset of S phase of the cell cycle [3, 4]. For this reason, TS is of great interest for the study of cell-cycle regulation. For TS in particular, control at the RNA transcriptional level has been inferred because the increase in TS activity prior to S phase in 3T6 cells synchronized by release from serum deprivation was blocked by treatment with actinomycin-D [3]. In Chinese hamster cells synchronized by release from isoleucine deprivation, an increase in TS activity prior to S phase was seen only in the nucleus, not in the cytoplasm, and was ascribed to assembly and translocation of the replitase from cytoplasm to nucleus [26]. The reasons for these differing results are not known. TS-deficient cells are an experimental tool for studying TS regulation. Introduction of human TS by DNA-mediated gene transfer into TS-deficient cells has already been accomplished and resulted in cell lines with interesting aberrations in TS activity and regulation [22]. With the successful isolation of cDNA for mouse TS [5] and genomic sequences for human TS [8], an analysis of the regulation of TS and control of the cell cycle, in general, will be possible. If gene regulation at the transcriptional level does indeed occur, TS-deficient cells will be useful for identifying and analyzing the controlling elements for such regulation.

Another important use of the TS-deficient cells described here is for the study of expression of human folate-dependent chromosomal fragile sites such as the site at Xq28 associated with X-linked mental retardation [10]. Extensive inferential evidence points to Td deprivation as a final common pathway for expression of these fragile sites [11]. Treating the cells with enzyme inhibitors or media that limit the activity of TS induces expression of the site; Td suppresses expression. The degree of Td deprivation achieved by FUdR treatment must therefore be a result of a balance between Td demand, a function of cell density, and Td supply, determined both by exogenous Td levels and the degree of inhibition of endogenous TMP synthesis. The expression of the fragile site in a hybrid deficient in TS following removal of Td from the medium provides direct evidence for Td deprivation as a necessary and sufficient condition for fragile site expression in this particular cell line. Hybrids containing a fragile X in a TS-deficient background simplify expression studies by eliminating the variables introduced by the use of antimetabolite drugs and thus reducing the problem to one of cellular demand for and exogenous supply of Td.

These hybrids are also an excellent system in which to study nucleotide pools during fragile site expression as well as the timing of replication of the fragile site DNA itself. They also should be ideal hosts for testing isolated cloned sequences for their ability to produce cytogenetic fragile site expression when introduced into these cells.

NOTE ADDED IN PROOF: Following acceptance of this manuscript, a paper entitled "Assignment of Human Gene Encoding Thymidylate Synthase to Chromosome 18 Using Interspecific Cell Hybrids between Thymidylate Synthase-Negative Mouse Mutant Cells and Human Diploid Fibroblasts" by Tadaaki Hori, Dai Ayusawa, Kimiko Shimizu, Hideki Koyama, and Takeshi Seno appeared in *Somatic Cell and Molecular Genetics* 11(3):277–284, 1985. The data of Hori et al. are derived from complementation of thymidine auxotrophy in their mouse TS-deficient cells by human chromosome 18 and are therefore similar to the results reported in this paper using our Chinese hamster TS-deficient cells. However, one of their hybrids appeared to retain its thymidine prototrophy despite undergoing an apparent spontaneous deletion of a portion of human 18q, suggesting that the structural gene may not be located in the region we are proposing based on quantitative Southern blotting. The spontaneous deletion hybrid of Hori et al. may be a translocation rather than a deletion although the translocated 18q was not seen cytogenetically. Resolution of this discrepancy awaits further analysis by in situ hybridization and by studies of other deletion 18 mutant cell lines.

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