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Thymidylate Synthase Gene Amplification in Fluorodeoxyuridine-Resistant Mouse Cell Lines

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SUMMARY

We have previously isolated fluorodeoxyuridine-resistant mouse fibroblast (LU3-7) and neuroblastoma (FUdR-R) cell lines that overproduce thymidylate synthase and the mRNA for this enzyme up to 50-fold as compared to the parental cell lines. We have also cloned cDNA corresponding to mouse thymidylate synthase mRNA into pBR322. In the present study, we used this cloned cDNA as a hybridization probe in Southern blot analysis of DNA from the parental and overproducing cell lines. These analyses showed that the thymidylate synthase gene is amplified 50–100-fold in LU3-7 cells and about 30-fold in FUdR-R cells when compared to the respective parental cells. The sizes of the restriction fragments were the same in the parental and overproducing cells of each type, suggesting that extensive rearrangements have not occurred in the vicinity of the thymidylate synthase gene during the amplification process. However, not all of the fragments in the parental cells were amplified in the overproducing cells, suggesting that there may be multiple genes or pseudogenes for the enzyme. Restriction fragment length polymorphisms were detected when analyzing DNA from several different mouse cell lines. When LU3-7 cells were grown in the absence of selective pressure, the level of thymidylate synthase overproduction and the number of copies of the thymidylate synthase gene decreased in parallel.

INTRODUCTION

Thymidylate synthase (EC 2.1.1.45) catalyzes the reductive methylation of deoxyuridylic acid to form thymidylic acid. The enzyme is indispensable in rapidly growing cells that are not provided with an alternate source of thymidine nucleotides. For this reason, TS[2] is an important target enzyme in cancer chemotherapy (1, 2). A variety of inhibitors of TS have been developed for use in chemotherapy, including fluorouracil and FdUrd (3). These analogues are converted to fluorodeoxyuridylic acid which, along with N^5,N^{10}-methylene tetrahydrofolate, forms a stoichiometric, covalent complex with the enzyme at the active site (4) and strongly inhibits its activity.

We have been studying the biochemical mechanisms for controlling TS enzyme activity in growth-stimulated mouse fibroblasts (5, 6). To facilitate these studies, we isolated a fluorodeoxyuridine-resistant mouse cell line (LU3-7) that overproduces TS and its mRNA by a factor of 50–100 (7). By several criteria, the overproduced enzyme appears to be the same as the normal enzyme. We have also isolated a FdUrd-resistant mouse C-46 neuroblastoma cell line (FUdR-R) that overproduces TS by a factor of about 12 (8, 9). We recently constructed a cDNA library corresponding to mRNA from the LU3-7 cells and identified several plasmids which contain sequences corresponding to almost the entire length of TS mRNA (10). In this study, we used the cloned TS cDNA as a hybridization probe in Southern blot analyses to study the structure and amplification of the TS gene in normal and overproducing cells.

MATERIALS AND METHODS

Cell culture. Cultures of mouse 3T6 cells (11) and the C-46 clone of C-1300 mouse neuroblastoma cells (8, 9) were maintained on plastic Petri dishes in the Dulbecco-Vogt modification of Eagle's medium (Gibco) containing 10% calf serum (Colorado Serum). The medium for the FdUrd-resistant fibroblasts was supplemented with 1 mM uridine, 1 mM cytidine, and 3 (for LU3-7 cells) or 0.3 μM (for UH.3-8 cells) FdUrd (7). FUdR-R cells were grown in medium supplemented with 0.4 μM FdUrd. The M50L3 cell line, the parental line for the LU3-7 cells, is a methotrexate-resistant line of 3T6 cells that overproduces dihydrofolate reductase 300-fold (12). M50L3 cells were grown in medium containing 50 μM methotrexate.

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[2] The abbreviations used are: TS, thymidylate synthase, FdUrd, fluorodeoxyuridine; kb, kilobase.

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DNA isolation. Exponentially growing cells were harvested and nuclei were prepared as described previously (13). Nuclei were prepared from Swiss mouse liver cells as described (14). Nuclei were lysed with sodium dodecyl sulfate, digested with RNase and then pronase, extracted gently with phenol/chloroform, and dialyzed exhaustively against 0.25 M NaCl, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.4) and then against 1 mM Tris-HCl (pH 7.4) and 0.1 mM EDTA. DNA concentration was determined by measuring absorbance at 260 nm.

Southern blot analysis. Restriction enzymes were purchased from New England Biolabs, Bethesda Reserach Laboratories, or International Biotechnologies, Inc. and used according to the manufacturers' instructions. Reactions contained 2–5 units of enzyme/μg of DNA and were incubated at 37° for 4–5 hr. Completion of the digestion reaction was monitored by determining the degree of digestion of λ phage DNA which was added to an aliquot of the digestion mixture and incubated in a separate tube. The restricted DNA was electrophoresed on a 1.0% agarose (Bethesda Research Laboratories, Inc.) gel in 5 mM sodium acetate, 1 mM EDTA, and 40 mM Tris-HCl, pH 7.9. DNA was transferred to nitrocellulose filters (Millipore) and probed essentially as described by Southern (15). The filters were prehybridized with 10 ml of 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.4), 1% sodium dodecyl sulfate, 5× Denhardt's solution (16) [Denhardt's solution contains 0.02% (w/v) each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll (M_r = 400,000)], 0.5 mg/ml poly(A), and 300 μg/ml of denatured sonicated salmon sperm DNA in a plastic bag at 65° for 20 hr. The prehybridization solution was then replaced with 10 ml of 2× SSC, 1% sodium dodecyl sulfate, 5× Denhardt's solution, 10% dextran sulfate (17) (Sigma), 150 μg/ml sonicated salmon sperm DNA, and 10^7 cpm of denatured nick-translated plasmid (10^8 cpm/μg) (18). Hybridization was at 65° for 20 hr.

RESULTS

Analysis of TS gene(s) in FdUrd-sensitive cells. To study the structure of the TS gene, we performed Southern blot analyses of DNA isolated from a variety of FdUrd-sensitive cell lines in which the TS gene was not expected to be amplified. The hybridization probe for these analyses was pMTS-4, which contains a 1.1-kb cDNA insert corresponding to almost the entire length of the predominant 1.3-kb TS mRNA (10). Fig. 1 shows that multiple restriction fragments were detected in each DNA preparation following digestion with *Eco*RI or *Hin*dIII. Since there are no sites for either of these enzymes within the TS cDNA insert (10), the multiple fragments may correspond to different exons of the TS gene or to other genes or pseudogenes with sequences closely related to the TS cDNA probe. Since the intensities of the fragments were similar in 3T6 and M50L3 cells, it is clear that the TS gene is not amplified in parallel with the dihydrofolate reductase gene. Therefore, the two genes are not tightly linked in mouse cells.

It was interesting to note that there were several differences in the restriction pattern when comparing DNA from various mouse cells, such as C-46, 3T6, and liver. The intensity and distribution of fragments were the same when comparing DNA from 3T6 and M50L3 cells, a methotrexate-resistant 3T6 cell line (12) that was the parental line for the LU3-7 cells. This was also true when 3T6 and M50L3 DNA were digested with other enzymes, such as *Pst*I and *Bgl*II (data not shown).

Hamster and human TS gene sequences were also readily detected in this analysis, indicating that the cloned mouse cDNA is a useful probe for TS sequences from a variety of higher eucaryotes. The intensities of

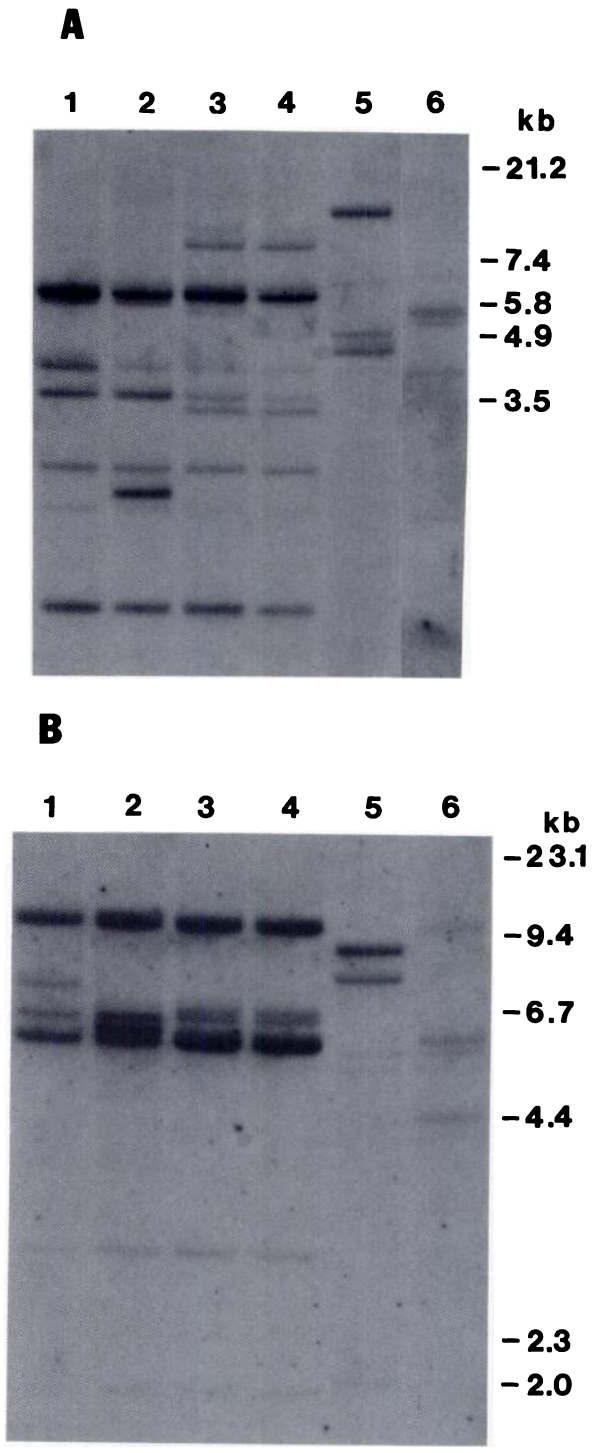

FIG. 1. *Southern blot analysis of TS gene structure in FdUrd-sensitive mouse, hamster, and human cells*

High molecular weight DNA (5 μg) was digested to completion with *Eco*RI (A) or *Hin*dIII (B) and electrophoresed on a 1% agarose gel. The DNA was transferred to nitrocellulose and probed with nick-translated pMTS-4. *Eco*RI- or *Hin*dIII-digested phage λ DNA served as molecular weight standard. Lane 1, C-46 (mouse neuroblastoma); lane 2, Swiss mouse liver; lane 3, 3T6 (mouse fibroblast); lane 4, M50L3 (dihydrofolate reductase-overproducing 3T6); lane 5, Chinese hamster ovary; lane 6, HeLa (human carcinoma).

the human fragments are significantly less than those of the mouse and hamster, suggesting that the mouse TS-coding sequence is more closely related to that of the hamster than to the human TS-coding sequence. The restriction fragment pattern for each organism was unique. Therefore, it should be possible to map the chromosomal location of the mouse TS gene by analyzing the restriction fragment pattern of DNA from various hamster-mouse hybrid cell lines containing known subsets of the mouse chromosomes (e.g., 19).

TS gene amplification in FdUrd-resistant cells. Blot analysis of DNA from LU3-7 cells revealed a striking increase in the hybridization intensity when compared to DNA from the parental cells (Fig. 2). All of the restriction fragments detected in LU3-7 DNA were also present in the M50L3 and 3T6 DNA. However, not all of the fragments present in the parental DNA were amplified in LU3-7 cells. In particular, the prominent band migrating at about 6.5 kb was present at approximately the same intensity in FdUrd-sensitive and resistant cell lines. Other bands that were fainter were also present at equal intensity in all DNA preparations. When identical filters were probed with nick-translated pBR322 DNA, no hybridization was detected. This eliminated the possibility that the extra bands were due to contamination of the digested DNA with small amounts of plasmid DNA which were being detected by the pMTS-4 probe.

To determine the extent of TS gene amplification more precisely, dilution analysis was performed. As shown in Fig. 2 (lanes 1–5) and confirmed by densitometric scanning, the intensity of the amplified bands was similar to that of the parental bands when the amount of LU3-7 DNA was reduced by a factor of 50–100, indicating a 50–100-fold amplification of the TS gene. This is the same as the -fold increase in TS enzyme (7) and mRNA (10) in LU3-7 cells as compared to 3T6 or M50L3 cells.

DNA from M50L3 and LU3-7 cells was also digested with *Hin*dIII, *Bgl*II or *Pst*I, which cut 0, 1, or 2 times within the thymidylate synthase cDNA insert, respectively (10). Three amplified restriction fragments were detected in DNA digested with *Hin*dIII (Fig. 3A), whereas five fragments were detected in *Bgl*II-digested DNA (Fig. 3B), and six fragments were detected in *Pst*I-digested DNA (Fig. 3C). Although some of the bands were faint, the original autoradiograms again showed that all of the amplified restriction fragments were present in the parental cell DNA, but not all of the parental fragments were amplified in the LU3-7 cells. *Bgl*II-digested M50L3 DNA clearly showed the presence of three intense bands (8, 6.5, and 4 kb) that were not amplified in LU3-7 cells.

Restriction analysis of DNA isolated from UH.3-8 cells, another FdUrd-resistant cell line derived from 3T6 cells instead of M50L3 cells (7), revealed that the TS gene was also amplified in this cell line (Fig. 4). Dilution analysis and densitometric scanning (similar to that in Fig. 2) showed that the TS gene was amplified 10–15-fold in UH.3-8 cells (data not shown). This is in line with the 19-fold overproduction of TS in UH.3-8 cells. The same pattern of amplified and unamplified fragments was observed in the UH.3-8 line as in LU3-7 cells.

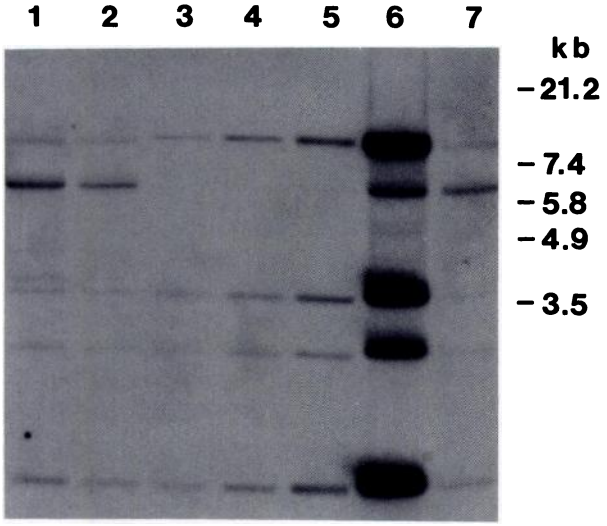

FIG. 2. *TS gene amplification in FdUrd-resistant LU3-7 cells*

DNA isolated from 3T6 and M50L3 cells and various dilutions of DNA isolated from LU3-7 cells were digested with *Eco*RI and analyzed as in Fig. 1. The intensity of the signal was compared to estimate the degree of gene amplification. Lane 1 contained 5 μg of 3T6 DNA. Lanes 2 and 7 contained 4 μg of M50L3 DNA. The remaining lanes contained LU3-7 DNA: lane 3, 0.05 μg; lane 4, 0.1 μg; lane 5, 0.2 μg; lane 6, 5 μg.

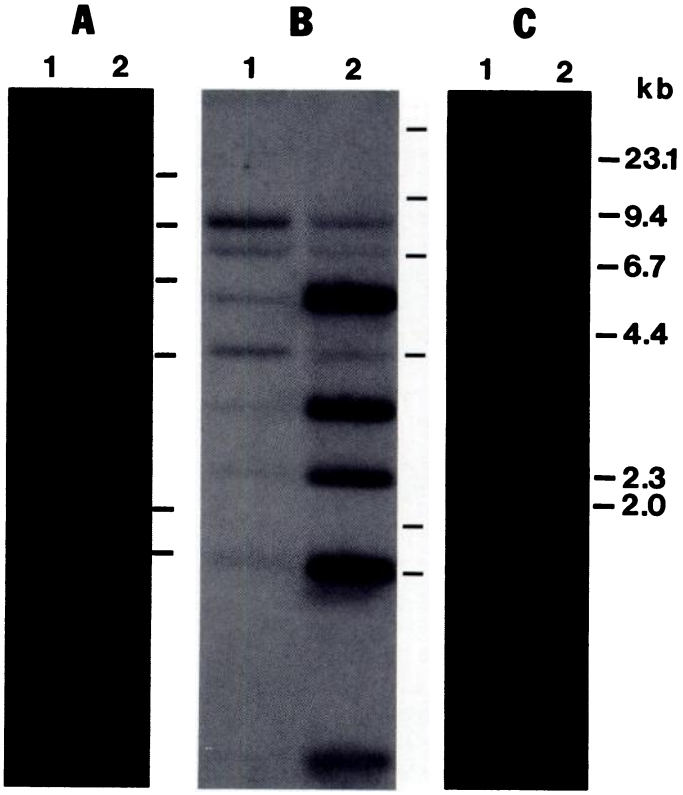

FIG. 3. *Analysis of the normal and amplified TS genes using other restriction enzymes*

DNA (5 μg) from M50L3 (lane 1) or LU3-7 (lane 2) cells was digested with *Hin*dIII (A), *Bgl*II (B), or *Pst*I (C) and analyzed as in Fig. 1. The bars represent the positions of molecular weight markers identified on C.

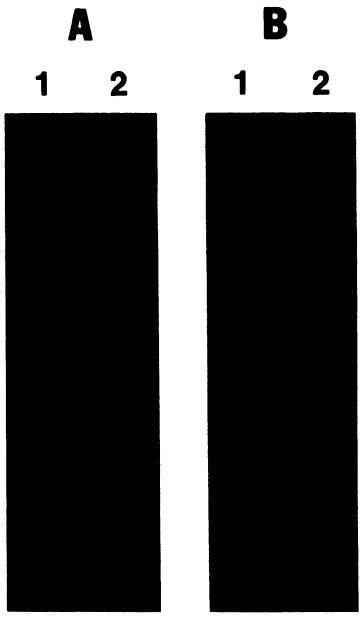

FIG. 4. *Amplification of TS gene in UH.3-8 cells*

DNA (5 μg) from LU3-7 cells (lane 1) or UH.3-8 cells (lane 2) was digested with *Eco*RI (A) or *Hind*III (B) and subjected to Southern blot analysis as in Fig. 1.

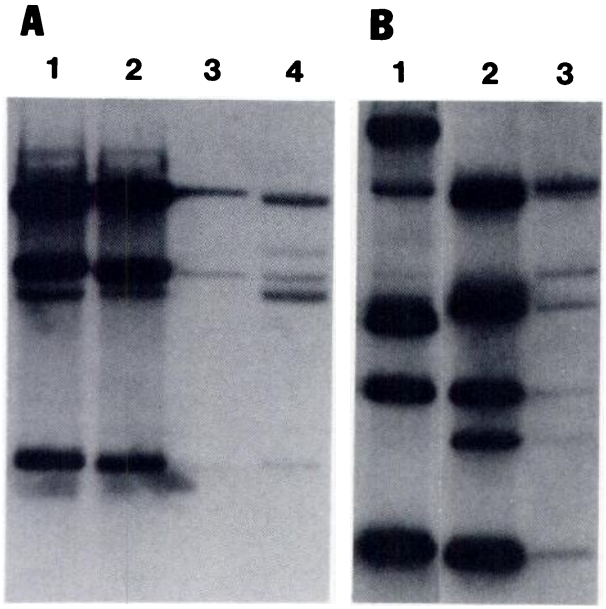

FIG. 5. *Amplification of TS gene in FUdR-R cells*

Five μg of DNA from LU3-7 (lanes 1), FUdR-R (lanes 2), C-46 (lanes A4 and B3), or 0.1 μg of DNA from FUdR-R cells (lane A3) were digested with *Hind*III (A) or *Eco*RI (B) and subjected to Southern blot analysis as in Fig. 1.

Therefore, it appears that the same TS gene was amplified in these two independently derived cell lines.

TS gene amplification in FdUrd-resistant mouse neuroblastoma cells. DNA isolated from mouse C-46 neuroblastoma and the FdUrd-resistant derivative (FUdR-R) was also analyzed by Southern blot analysis and densitometry. Fig. 5 shows that the TS gene was amplified about 30-fold in FUdR-R cells as compared to the parental line. This is somewhat greater than the 12-fold increase in the TS enzyme level, as measured by antibody

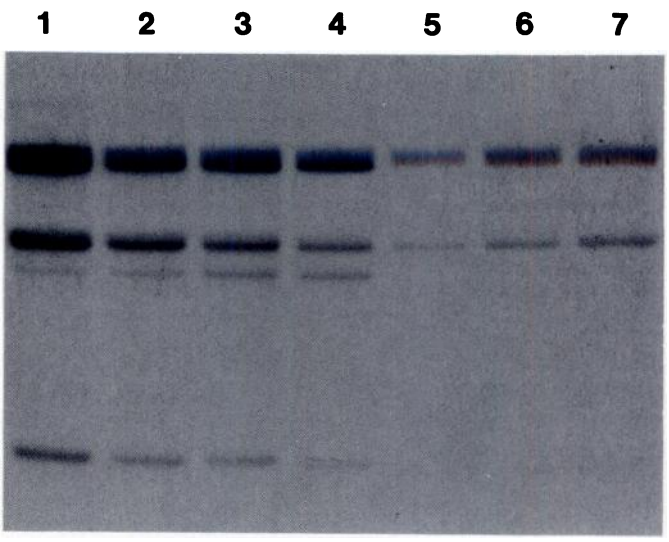

FIG. 6. *Loss of amplified TS genes when LU3-7 cells are grown in the absence of FdUrd*

DNA was prepared from control LU3-7 cells or from LU3-7 cells maintained in medium lacking FdUrd for the indicated times. The DNA (5 μg) was digested with *Hind*III and analyzed as in Fig. 1. Lane 1, 0 months; lane 2, 1 month; lane 3, 2 months; lane 4, 3 months; lane 5, 0.1 μg of control LU3-7 DNA; lane 6, 0.25 μg of control LU3-7 DNA; lane 7, 0.5 μg of control LU3-7 DNA.

precipitation or stoichiometric inactivation with 5-fluorodeoxyuridylic acid (8, 9). Although the same bands were present in the parental as in the overproducing cell line, not all of the bands were amplified. When the DNA was digested with *Hind*III (Fig. 5A), the same pattern of amplified bands was observed in DNA isolated from LU3-7 and FUdR-R cells. However, when the DNA was digested with *Eco*RI (Fig. 5B), the patterns were somewhat different. This is probably due to restriction site polymorphisms in the TS gene in the two different cell lines (see Fig. 1).

Loss of amplified genes in the absence of selective pressure. When LU3-7 or FUdR-R cells are grown in medium lacking FdUrd, their extent of overproduction of TS and their resistance to this drug gradually decrease (7, 8). The half-life of the decrease is about 30 days in both cell lines. The decrease in TS overproduction could be due to a decrease in gene copy number. Alternatively, the number of TS genes might remain constant but the level of expression might decrease due to other mechanisms. Fig. 6 shows that the degree of TS gene amplification decreased to approximately the same extent as the specific activity of the enzyme when LU3-7 cells were grown in medium lacking FdUrd. Comparison of lanes 4 and 7 by densitometry showed that the extent of TS gene amplification decreased about 5–10-fold after 3 months. Lanes 1–4 show that the intensites of the amplified restriction fragments decreased in parallel. However, the intensity of the unamplified 5.5-kb restriction fragment remained constant.

DISCUSSION

These results clearly show that the TS gene is amplified in FdUrd-resistant LU3-7 and FUdR-R cells. Since there is a parallel increase in the amount and activity of

TS (7), TS mRNA (10), and the number of copies of the TS gene in LU3-7 cells, it is likely that most if not all of the amplified genes are transcriptionally active in this resistant cell line. Thus, LU3-7 cells are similar to other cell lines that have achieved resistance to other toxic agents by virtue of parallel increases in the target enzyme and its gene (reviewed in Refs. 20 and 21). In addition, TS gene expression is regulated following growth stimulation in the same manner in the LU3-7 cells as in the parental cells (22). Therefore, amplification of the TS gene has not impaired its ability to be regulated.

In contrast, in FUdR-R cells, there is a 30-fold increase in the number of copies of the TS gene, but only a 12-fold increase in the amount of TS enzyme (8, 9). This suggests differential transcriptional efficiency or stability of TS enzyme or mRNA in the overproducing as compared to the parental neuroblastoma cell lines.

Only a subset of the restriction fragments that were detected in the parental cell line were amplified in LU3-7 or FUdR-R cells. One possible explanation is that there is more than one functional TS gene in mouse cells. Although it is probable that most enzymes are encoded by a single gene, Melera and coworkers (23) showed that, in hamster cells, there are two genes for dihydrofolate reductase that code for slightly different forms of the enzyme. A second possibility is that the amplified fragments represent the functional gene and that the unamplified fragments contain TS pseudogenes. Processed pseudogenes for dihydrofolate reductase that closely resemble dihydrofolate reductase mRNA have been found in DNA from normal human cells (24, 25). It has been suggested that processed pseudogenes may be particularly prevalent for enzymes or proteins that are expressed in germ line tissues (26), as would be true in the case of TS. The fragments that are not amplified are among the most intense bands detected on the Southern blots of the parental DNA (e.g., Figs. 1A and 3B). This would be expected if the unamplified fragments contain processed TS pesudogenes since all of the exon segments that hybridize with the probe could be present within a single fragment. A third possibility is that the probe is detecting other genes or DNA sequences that show some homology to portions of the TS cDNA probe.

Amplified genes for enzymes have been localized either to homogeneously staining chromosomal regions (27) or to double minute chromosomes (28). The former are relatively stable when cells are grown in the absence of selective pressure while the latter are relatively unstable. Since the TS overproduction trait (7) and the number of copies of the TS gene decrease when LU3-7 cells are grown in medium lacking FdUrd, it is likely that the amplified TS genes are found on double minute chromosomes. This will be difficult to confirm by karyotype analysis since the parental cell line (M50L3) already contains double minute chromosomes.[3] However hybridization analysis of purified double minute chromosomes should clarify this issue.

A variety of restriction fragment length polymorphisms were detected when comparing DNA from various mouse cells. When comparing Figs. 1, 2, 3, and 5, it is clear that some of these polymorphisms were within fragments containing portions of a functional TS gene. This probably indicates that the intervening sequences or gene-flanking sequences have diverged fairly rapidly, either as a result of nucleotide substitutions or DNA rearrangements. It will be interesting to determine if the coding sequences have also diverged in different mouse species and if the mouse TS cDNA or amino acid sequences show homology to procaryotic TS sequences. Detailed analysis of the mouse TS gene sequences will be possible once the gene(s) has been cloned (in progress). The molecular cloning of the human TS gene has recently been reported (29).

[3] M. Collins and L. F. Johnson, unpublished observations.

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