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Induction, by Thymidylate Stress, of Genetic Recombination as Evidenced by Deletion of a Transferred Genetic Marker in Mouse FM3A Cells

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Studies were made on the genetic consequences of methotrexate-directed thymidylate stress, focusing attention on a human thymidylate synthase gene that was introduced as a heterologous genetic marker into mouse thymidylate synthase-negative mutant cells. Thymidylate stress induced thymidylate synthase-negative segregants with concomitant loss of human thymidylate synthase activity with frequencies 1 to 2 orders of magnitude higher than the uninduced spontaneous level in some but not all transformant lines. Induction of the segregants was suppressed almost completely by cycloheximide and partially by caffeine. Thymidylate stress did not, however, induce mutations, as determined by measuring resistance to ouabain or 6-thioguanine. Thymidylate synthase-negative segregants were also induced by other means such as bromodeoxyuridine treatment and X-ray irradiation. In each of the synthase-negative segregants induced by thymidylate stress, a DNA segment including almost the whole coding region of the transferred human thymidylate synthase gene was deleted in a very specific manner, as shown by Southern blot analysis with a human *Alu* sequence and a human thymidylate synthase cDNA as probes. In the segregants that emerged spontaneously at low frequency, the entire transferred genetic marker was lost. In the segregants induced by X-ray irradiation, structural alterations of the genetic marker were random. These results show that thymidylate stress is a physiological factor that provokes the instability of this exogenously incorporated DNA in some specific manner and produces nonrandom genetic recombination in mammalian cells.

Thymidylate synthase (TS, EC 2.1.1.45) is a key enzyme in DNA replication because it catalyzes the only de novo pathway for supplying dTTP and is essential in regulating the balanced supply of the four DNA precursors. Since its activity is proportional to the proliferative potential of cells, this enzyme has been studied as a target of several widely used cancer chemotherapeutic agents.

TS-negative (TS^-) mutants of mouse FM3A cells undergo rapid cell death, called thymineless death, upon thymidine starvation with concomitant induction of a particular type of DNA double-strand breaks (DSBs) (4). Thymidine starvation of the wild-type cells can be mimicked by use of drugs, including cancer chemotherapeutic agents that block the production of reduced folate or thymidylate. The genetic consequences of thymidylate stress in these cells are of particular concern in view of the following facts. (i) Thymidine starvation of TS^- mouse mutant cells provokes sister chromatid exchanges and chromosome aberrations closely coupled with DNA replication (18, 19). (ii) Thymidylate stress induces mutation and recombination in procaryotes (11, 35) and recombination but not mutation in *Saccharomyces cerevisiae* (7, 25). In mammalian cells, severe thymidylate stress is not mutagenic, whereas mild thymidylate stress (21) or excess supply of thymidylate induces mutation (9, 15). (iii) Thymidylate stress induces both heritable and constitutive fragile sites on human chromosomes (37, 45). Recent findings of a correlation between the fragile sites and break points involved in chromosomal rearrangements in some neoplastic cells suggest that thymidylate stress may be an in vivo genetic determinant that directs specific chromosome rearrangements at the most susceptible sites (27, 44, 45). We have already confirmed that mild thymidine starvation preferentially induces expression of a heritable fragile site on the affected human X chromosome introduced into TS^- mouse mutant cells by cell fusion (17).

In this work, we examined whether thymidylate stress provokes a genetic alteration in a certain genetic marker as it does chromosomal breaks and rearrangements. For this purpose, we constructed mouse cell transformant lines in which the human TS gene was stably integrated. We observed some nonrandom genetic alterations that occurred in the introduced foreign gene in the transformants as a consequence of thymidylate stress and compared them with the alteration that occurred spontaneously and on X-ray irradiation or bromodeoxyuridine (BrdUrd) treatment.

MATERIALS AND METHODS

Cell line and culture conditions. A primary prototrophic transformant clone FS*thy*11/*thy*H-1 (11-1) was derived from a thymidine auxotrophic mutant (TS^-) FS*thy*11 of mouse FM3A cells (1) after DNA-mediated transfer of the human TS gene with total human DNA as described previously (3). Two secondary transformants named C0 and D3 were constructed similarly from FS*thy*11 with DNA of the primary transformant 11-1 (3). The cells were maintained in ES medium (22) (Nissui Seiyaku Co., Tokyo) containing 2% dialyzed fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.). When indicated, 10 μM thymidine was added to cultures to support normal growth of thymidine-auxotrophic cells. All cells were cultured in plastic petri dishes (NUNCRON, Denmark) at 37°C in an incubator under 5% CO_2 in air at 95% humidity.

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Induction of genetic variants by thymidylate stress, BrdUrd, and X-ray irradiation. Exponentially growing transformant cells cultured in thymidine-free medium were diluted to a density of 10^5 cells per ml, and samples of the cell suspension were subjected to thymidylate stress. For this, they were incubated for the indicated times in the presence of 1 μM methotrexate (Sigma Chemical Co., St. Louis, Mo.) and 100 μM hypoxanthine, washed twice with ES medium supplemented with 2% fetal calf serum and 10 μM thymidine, and then cultured for 1 day for the expression of altered phenotypes in ES medium supplemented with 2% fetal calf serum, 10 μM thymidine, 25 mM hypoxanthine, and 1 μM 5-methyltetrahydrofolate (Sigma) and for 4 days in ES medium supplemented with 2% fetal calf serum and 10 μM thymidine. The cells were then plated on selective plates as specified below.

For the induction of mutations by BrdUrd, portions of the above cell suspension were cultured in the presence of BrdUrd for 24 h. The cells were washed once with ES medium supplemented with 2% fetal calf serum and 10 μM thymidine, cultured in the same medium, and then plated on selective plates.

For X-ray irradiation, cells were suspended at a density of 10^5 cells per ml in ES medium containing 2% fetal calf serum and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (pH 7.2). Cells were irradiated on ice at a dose rate of 100 rads per min at 225 kV and 17.5 mA. Then the cells were cultured for 20 h in ES medium supplemented with 2% fetal calf serum and 10 μM thymidine to allow recovery and plated on selective plates.

Determination of survival. The effects of thymidylate stress on viability and growth of cells were determined in two ways. First, cells were starved of thymidylate, washed with medium containing 10 μM thymidine, 100 μM hypoxanthine, and 1 μM 5-methyltetrahydrofolate, and cultured in the same medium at an initial density of 10^4 cells per ml. At intervals, cells were counted in a Coulter counter (model MHR; Coulter Electronics, Inc., Hialeah, Fla.).

Second, cells starved of thymidylate were washed twice with the above medium and plated on normal agarose medium consisting of ES medium solidified with 0.35% agarose (Seakem, Rockland, Maine) and supplemented with 5% fetal calf serum, 10 μM thymidine, 100 μM hypoxanthine, and 1 μM 5-methyltetrahydrofolate as described previously (1, 4). After incubation for 1 week, visible colonies were counted.

X-irradiated cells were immediately plated on the surface of ES agarose medium supplemented with 5% fetal calf serum and 10 μM thymidine. After incubation for 1 week, visible colonies were counted.

Selection of genetic variants. Cells exposed to thymidylate stress, BrdUrd, or X-ray irradiation were allowed to recover from damage and to express phenotypes by culture for 5 or more days in growth medium before being plated on selective agarose plates. TS^- clones were selected on ES agarose medium supplemented with 5% dialyzed fetal calf serum, 10 μM thymidine, 0.3 μM methotrexate, and 1 μM 5-methyltetrahydrofolate; visible colonies formed after incubation for 7 to 10 days were scored and picked up for further characterization as described previously (1, 2). 6-Thioguanine-resistant (TG^r) and ouabain-resistant (Oua^r) mutants were selected on ES agarose medium supplemented with 5% fetal calf serum, 10 μM thymidine, and either 10 μM 6-thioguanine (Sigma) or 2 mM ouabain (Sigma) as described previously (1, 21).

Southern blot analysis. High-molecular-weight DNAs were digested with restriction endonuclease *Eco*RI. Fragments were resolved by electrophoresis on 1.0% agarose gel, transferred to nitrocellulose filters (Schleicher & Schüll, West Germany), and hybridized to nick-translated DNA probes (3). Two kinds of DNA probes were used. One was a recombinant plasmid BLUR8 that contained an interspersed human middle-repetitive *Alu* sequence to detect human-specific DNA fragments as described previously (3). The other was a fragment of about 1.1 kilobases (kb) of the cDNA insert derived from a recombinant plasmid containing functional human TS cDNA, pcHTS-1. This fragment was excised from the plasmid by restriction enzymes *Bgl*I and *Hpa*I and contained almost the entire coding sequence and a portion of the 3′ untranslated region (5, 40). DNA probes were nick translated with [α-^{32}P]dCTP with a nick translation kit (Amersham Corp., Arlington Heights, Ill.) to a specific activity of 2×10^9 cpm/μg of DNA. Hybridization and washing conditions were as described in detail previously (3, 5).

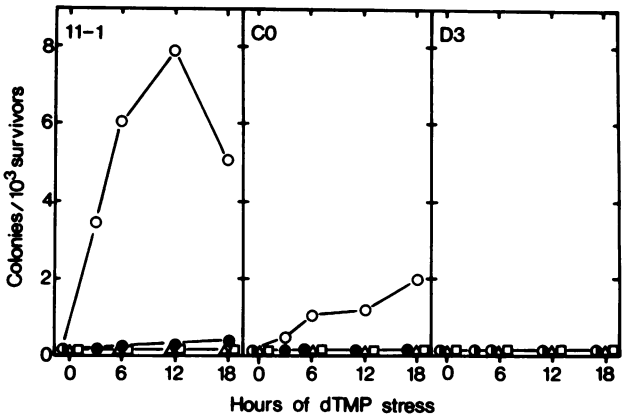

FIG. 1. Induction of TS^- clones by thymidylate stress in a primary transformant, 11-1, and its two secondary transformants, C0 and D3. Cells were exposed to thymidylate stress for the indicated times and processed as described in Materials and Methods. Then the cells were plated onto 5 ml of three kinds of selective agarose medium as described in Materials and Methods. After incubation for 10 days, colonies were scored. Open and closed symbols indicate the absence and presence of thymidine, respectively, during methotrexate-mediated thymidylate stress. Symbols: ○ and ●, TS^- clones; △, TG^r clones; □, Oua^r clones.

RESULTS

Induction of genetic changes by thymidylate stress. We first tested whether thymidylate stress induces genetic changes on the introduced human TS gene in a primary transformant, 11-1, and its two secondary transformants, C0 and D3 (3, 39). Thymidylate stress induced a considerable number of TS^- clones in primary transformant 11-1, fewer in secondary transformant C0, and none in secondary transformant D3 (Fig. 1). The induced levels were about 100-fold higher in 11-1 cells 12 h after exposure and about 50-fold higher in C0 cells 18 h after exposure than the respective uninduced levels. No induction of TS^- clones was observed when thymidine was added to culture during methotrexate-directed thymidylate stress. As described previously (1, 2) and in Materials and Methods, TS^- clones were selected by their high level of methotrexate resistance (MTX^r) in the presence of reduced folate. We examined at least 50 independent MTX^r colonies and confirmed that they were all

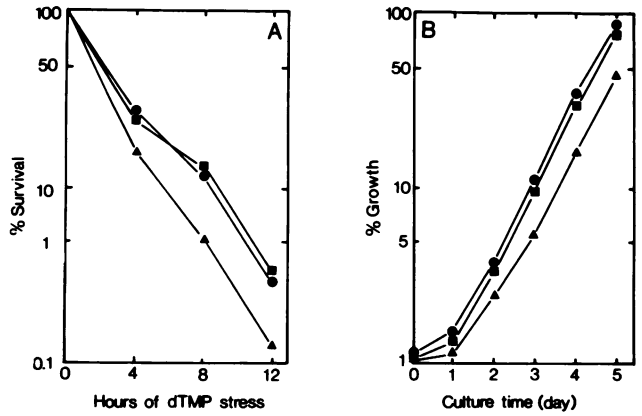

FIG. 2. Effects of thymidylate stress on survival (A) and growth (B) in the C0 transformant and its two TS⁻ segregants induced by thymidylate stress. (A) Cells were exposed to thymidylate stress for the times indicated, washed, and plated on normal agarose medium as described in Materials and Methods. (B) Cells were exposed to thymidylate stress for 12 h, washed, and cultured in normal growth medium containing 10 μM thymidine and 1 μM 5-methyltetrahydrofolate. At intervals, cells were counted, and cell numbers were plotted against time of culture. Symbols: ●, secondary transformant C0; ▲ and ■, independent TS⁻ segregants, clones 1 and 2, respectively.

thymidine auxotrophs (TS⁻) and showed normal sensitivity to methotrexate in the absence of reduced folate. The appearance of TS⁻ clones could not be due to point mutations because severe thymidylate stress did not induce mutations (21). In fact, TG^r and Oua^r clones were not induced significantly in any of the three transformant lines (Fig. 1).

In D3 cells, TS⁻ clones were not induced beyond a background frequency of 10^{-7} per cell. This frequency is close to that expected for autosomal recessive mutation.

Genesis of TS⁻ clones under thymidylate stress. Since thymidylate stress causes marked cell death and transformant clones 11-1 and C0 produced TS⁻ clones spontaneously at frequencies of 10^{-5} to 10^{-4} per cell in normal growth conditions in the presence of thymidine, the question arises of whether this high level of induction of TS⁻ clones by thymidylate stress could be the result of selective enrichment of preexisting TS⁻ cells by methotrexate-induced thymidylate stress rather than actual induction of TS⁻ cells. Although the above possibility is less likely because the cells had been maintained in the absence of thymidine, under which conditions TS⁻ cells should not be accumulated in the culture, but rather should be eliminated, we tested the effect of thymidylate stress on the C0 line and its two independently derived TS⁻ clones in two ways. First, we tested the effect of methotrexate-derived thymidylate stress on viability. The stress conditions we employed had similar killing effects on the three cell lines (Fig. 2A), thus excluding the possibility that TS⁻ cells survive preferentially during thymidylate stress. Second, we tested the growth rates of these cell lines after release from thymidylate stress for 12 h. The three cell lines grew similarly (Fig. 2B).

In fact, thymidylate stress resulted in a net increase in TS⁻ cells rather than an increase in the relative frequency of TS⁻ cells among survivors. For example, on exposure to thymidylate stress for 6 h about 30% of the 11-1 and C0 transformants survived and then produced about 600 and 100 TS⁻ cells per 10^5 survivors, respectively. Therefore, the actual numbers of TS⁻ cells obtained per 10^5 treated cells were about 180 and 30, respectively. These values are 10-fold higher than those of spontaneous TS⁻ cells (5×10^{-5} to 10×10^{-5} for 11-1 cells and 1×10^{-5} to 5×10^{-5} for C0 cells). Taken together, these results are consistent, although indirectly, with the possibility that thymidylate stress induces TS⁻ cells.

Induction of TS⁻ clones by BrdUrd and X rays. Since thymidylate stress did not induce mutations in the transformant lines, measured with Oua^r and TG^r as genetic markers (Fig. 1) as a control experiment, we examined the induction of mutations by BrdUrd, a well-known mutagen in mammalian cells, in the transformant C0. In this case, MTX^r, Oua^r, and TG^r mutants were all induced dose dependently (data not shown). However, in contrast to the MTX^r (TS⁻) clones induced by thymidylate stress, the MTX^r clones induced by BrdUrd were not all TS⁻ clones, but consisted of TS⁻ clones (80%) and thymidine-prototrophic MTX^r clones (20%). In the presence of 10 μM BrdUrd, MTX^r, Oua^r, and TG^r clones appeared with frequencies of 6.0×10^{-4}, 1.1×10^{-4}, and 3.9×10^{-4} per surviving cell, respectively. These values were 38-fold, 110-fold, and 21-fold the uninduced levels, respectively. Since BrdUrd induces point mutation as a consequence of pool imbalance of DNA precursors (9, 15, 21), it is reasonable to assume that all of the mutation phenotypes were induced by single events. The thymidine-prototrophic MTX^r clones are thus most likely due to alteration in the dihydrofolate reductase gene rather than in transport of methotrexate (1). These data indicate that C0 cells show a normal response to induction of mutations by BrdUrd and suggest that these cells have a single copy of the functional human TS gene.

We also tested the inductions of TS⁻ clones of the C0 and D3 transformant lines by X-ray irradiation, since X-ray irradiation induces random DNA DSBs, whereas thymidylate stress induces a particular type of DSB (4). Although thymidylate stress did not induce TS⁻ clones in the D3 line (Fig. 1), X-ray irradiation induced TS⁻ clones markedly and dose dependently in both cell lines. For example, X-ray doses of 750 rads induced 300 and 150 TS⁻ clones per 10^5 survivors of the C0 and D3 lines, respectively. X-ray irradiation did not induce TG^r or Oua^r clones significantly in either line. At present we do not know the reason for these different inductions of TS⁻ segregants of the D3 line by X rays and thymidylate stress. It may be related to where and how the human TS gene is integrated into the host genome in the two transformants, especially in terms of scheduled DNA replication.

Effects of various agents on induction by thymidylate stress of TS⁻ clones. We tested the effects of various agents on induction of TS⁻ clones of the C0 line by thymidylate stress. Cycloheximide blocked induction of TS⁻ clones (Fig. 3A) and cell death when it was present during thymidylate stress. These results were consistent with earlier findings that cycloheximide blocked induction of thymineless death, DNA DSBs, and chromosome aberrations during thymidine deprivation of TS⁻ mouse mutant cells (4, 19). When cycloheximide was present during recovery conditions after exposure to thymidylate stress, cell death was increased, whereas the frequency of induction of TS⁻ clones was unaltered. On X-ray irradiation, cycloheximide reduced induction of TS⁻ clones by 30 to 40% (Fig. 3B) and slightly increased cell death.

The presence of caffeine during thymidylate stress of C0 cells reduced the induction of TS⁻ cells about 50% (Fig. 3A) but enhanced cell death fivefold. Caffeine is thought to inhibit postreplication repair of various DNA damages in

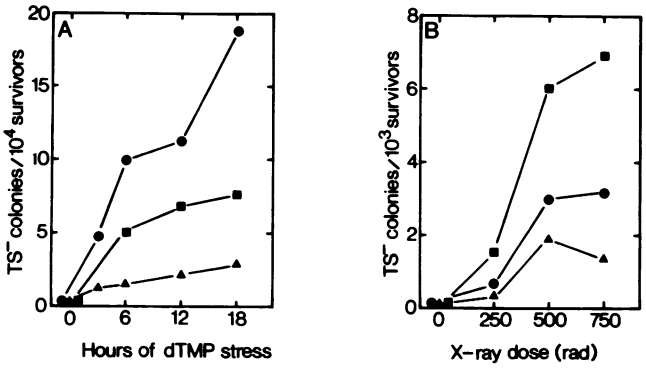

FIG. 3. Effects of cycloheximide and caffeine on induction of TS⁻ clones upon (A) thymidylate stress and (B) X-ray irradiation of C0 cells. Cells were starved of thymidylate or irradiated by X-ray as described in Materials and Methods. Cycloheximide (10 μg/ml) was added to the cells only during thymidylate stress in A and during recovery incubation in B. Caffeine (2 mM) was present during thymidylate stress and also for 20 h in the following recovery period in A and during recovery incubation in B. Symbols: ●, no addition; ▲, cycloheximide; ■, caffeine.

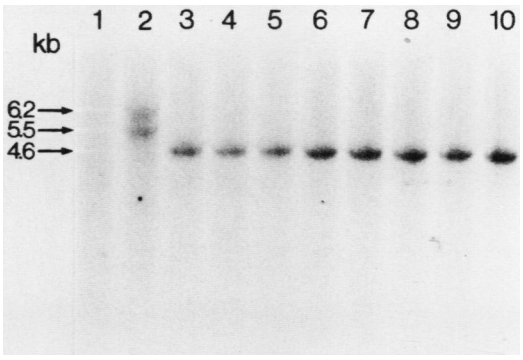

FIG. 5. Southern blot analysis of the human TS gene in the TS⁻ segregants induced by thymidylate stress with a human *Alu* sequence as a probe. Samples (20 μg) of high-molecular-weight DNA of each clone were digested completely with *Eco*RI endonuclease, and the digests were subjected to electrophoresis on 1% agarose gel, blotted onto a nitrocellulose filter, and probed with ^{32}P-labeled recombinant plasmid DNA containing a human *Alu* sequence as described previously (3). Lanes: 1 and 2, a recipient mouse TS⁻ mutant and the transformant C0, respectively; 3 through 10, independent TS⁻ segregants, clones 1 through 8, respectively, obtained from C0 cells after 6 h of thymidylate stress.

rodent cells (28) and has been shown to inhibit purine and pyrimidine biosyntheses (33). On X-ray induction, caffeine enhanced the induction of TS⁻ clones twofold (Fig. 3B) with concomitant stimulation of cell death by removing an initial shoulder on the survival curve but not by changing the slope (data not shown).

Several other biologically active substances were also tested. The tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate significantly suppressed induction of TS⁻ cells and significantly increased cell death. This tumor promoter is known to stimulate gene amplification (42), which is one form of genetic recombination. Antipain, a peptide inhibitor of certain proteases, had no significant effect.

Southern blot analysis of TS⁻ segregants. We examined structural changes in the transferred human TS gene in the TS⁻ segregants of C0 cells after phenotypic change from TS⁺ to TS⁻. The human TS gene present in C0 cells contains 6.2-, 5.5-, 1.6-, 5.4-, and 6.2-kb *Eco*RI fragments in this order from the 5′ to 3′ end (Fig. 4) (39; unpublished data). All of these fragments hybridized to full-length cDNA for human TS, and all but the 1.6-kb fragment hybridized to a human interspersed middle repetitive *Alu* sequence. The *Alu* sequence was not detected in any *Eco*RI fragments other than these in C0 cells. Consequently, Southern blot analysis of DNA from C0 cells gave two apparent bands of 6.2 and 5.5 kb, each containing two overlapping bands when the *Alu* sequence was used as a probe (Fig. 5, lane 2).

First, we examined eight independent TS⁻ clones isolated after exposure of cells to thymidylate stress for 6 h. In all of the TS⁻ segregants, the four *Eco*RI bands hybridizing to the *Alu* sequence in C0 cells were lost; instead, a new 4.6-kb band was observed (Fig. 5). These results show unequivocally that a particular type of DNA deletion was induced in the transferred human TS gene. Then we repeated the experiments with a 1.1-kb fragment of human TS cDNA as described in Materials and Methods instead of the *Alu* sequence as a probe. In C0 cells, the human TS cDNA probe apparently hybridized to the 6.2-, 5.5-, and 1.6-*Eco*RI fragments derived from the transferred human TS gene and also cross-hybridized to several *Eco*RI fragments of the endogenous mouse TS gene (Fig. 6). The three bands representing the entire coding region of the transferred human TS gene were deleted in all four independently induced TS⁻ segregants without affecting the structure of the endogenous mouse homolog. In comparison of these bands in C0 cells with those in the TS⁻ segregants as well as the recipient cells, the 6.2-kb band contained both human and mouse DNA fragments. In the case of three X ray-induced TS⁻ segregants, one segregant gave a new *Eco*RI band corresponding to about 5 kb, but the two bands detected in C0 cells were lost when probed by the *Alu* sequence. The other two segregants gave no bands hybridizable with the *Alu* sequence (data not shown).

Second, we examined eight independent TS⁻ clones that emerged spontaneously in C0 cells (Fig. 7). In contrast to the results with the induced TS⁻ clones (Fig. 5), no *Alu* sequence was detected in any of these eight TS⁻ clones, indicating that the entire human TS gene was lost in these cells. In cultured mammalian cells, chromosome loss is one

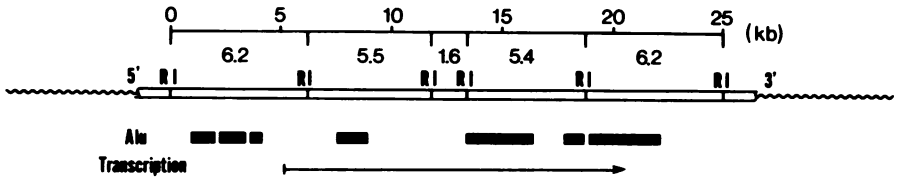

FIG. 4. Structure of the human TS gene integrated into the genome of mouse TS⁻ mutant cells. The structure is derived from previous data (39) and unpublished data. Symbols: ▭, restriction enzyme *Eco*RI cleavage map of the human TS gene present in C0 cells; ⁓, mouse DNA flanking the integrated human TS gene; ▬ (under the map), regions containing at least one copy of the human *Alu* sequence. The bottom arrow indicates the approximate region transcribed.

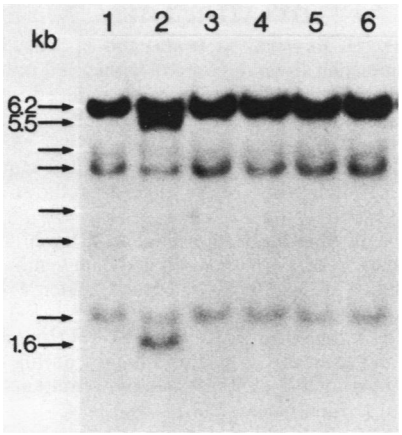

FIG. 6. Southern blot analysis of the human TS gene in the TS$^-$ segregants induced by thymidylate stress with a TS cDNA clone as a probe. Experimental conditions were as described in the legend to Fig. 5, except that human TS cDNA was used as the probe. Lanes: 1 and 2, a recipient mouse TS$^-$ mutant and the transformant C0, respectively; 3 through 6, the independent TS$^-$ clones 1 through 4, respectively, as described in the legend to Fig. 5.

of the most frequent mechanisms by which recessive traits are expressed for genes present heterozygously or hemizygously (12, 43). In fact, in mouse FM3A cells chromosome loss occurs at a frequency of 10^{-5} to 10^{-4} per cell (1). Thus chromosome loss may well be responsible for the occurrence of spontaneous TS$^-$ segregants in this study. This interpretation is consistent with the conclusion that C0 cells probably carry one copy of the human TS gene, as deduced from the frequencies of mutations induced by thymidylate stress and BrdUrd on the transferred human TS gene and the profiles of the Southern blot hybridization experiments (Fig. 5 through 7).

The results clearly show that the TS$^-$ segregants induced by thymidylate stress, those induced by X rays, and those generated spontaneously were produced by different mechanisms. The results also support the conclusion stated above that the TS$^-$ clones were actually induced by thymidylate stress, but not originated from selective enrichment of preexisting spontaneous TS$^-$ cells.

DISCUSSION

The present study showed that thymidylate stress was a strong initiator of recombination, at least for the introduced foreign gene system in mammalian cells. At present time this conclusion may not apply to bona fide chromosomal loci in general. It should be mentioned that thymidylate stress induced TS$^-$ cells in the primary transformant 11-1 and in the secondary transformant C0, but not in D3 (Fig. 1). It may well be that the induction of TS$^-$ cells by deletion is the result of a specific arrangement in the introduced DNA. However, this induction of deletion is consistent with the observation that thymidylate stress induces various types of chromosome aberrations and sister chromatid exchanges in mouse FM3A cells and induces expression of fragile sites on human chromosomes, some of which are postulated to be associated with specific chromosome rearrangement in tumor cells (27, 44, 45). Genetic rearrangement of the transferred human TS gene induced by thymidylate stress was of a unique type in which a particular portion covering almost the entire coding region was deleted. This implies that there is a particular type of DNA arrangement that is prone to induction of DNA DSBs and recombination in the transferred TS gene. Recombination between the transferred human TS gene and the endogenous mouse homolog is unlikely, because no structural change was observed in the mouse TS gene (Fig. 6). Since there are at least seven *Alu* sequences in the transferred human TS gene (Fig. 4), homologous recombination between these repeats might be involved in the deletion in association with the induction of DNA DSBs. This possibility is supported by a recent finding that the abnormal molecular size of the low-density lipoprotein receptor in a patient with familial hypercholesterolemia was due to a deletion in the receptor gene which appeared to be caused by a novel intragenic recombination between two *Alu* sequence that were oriented in opposite directions (29). However, the orientations of the *Alu* sequences in the human TS gene are not known. In *S. cerevisiae*, it is reported that thymidylate stress mediated by an antifolate drug induces intrachromosomal reciprocal recombination between the duplicated genes (B. A. Kunz, personal communication) and mitotic recombination as well (25). Another possible explanation is that recombination events in the transferred human TS gene occur by simple breakage and reunion, unrelated to specific sequence elements, in a manner which appears to be responsible for several deletions in the β-like globin gene cluster (41) and the collagen genes (8, 13). If so, the induction of DNA DSBs at a specific site should enhance the frequency of recombination events.

In mammalian cell systems, homologous recombination between two kinds of plasmids each containing a heteroallelic defect has been extensively studied extrachromosomally and intrachromosomally, for example, in the herpes simplex virus thymidine kinase gene and the *Escherichia coli* neomycin resistance gene (10, 16, 23, 24, 30, 32, 34, 36). In these cases homologous recombination between overlapping sequences is expected to produce a functional wild-type gene. In some of these experiments, introduction of a DSB into the donor plasmid enhanced the frequency of homologous recombination 10 to 100 times (10, 24). In yeast, the DNA double strand repair model is proposed to explain the mechanisms of homologous recombination. This model is based on the findings that homologous recombination and accompanying gene conversion between a donor gene carried by a plasmid and a recipient targeted allele on a

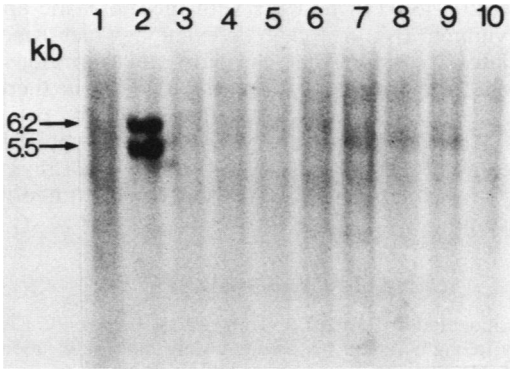

FIG. 7. Southern blot analysis of the human TS gene in the spontaneous TS$^-$ segregants with a human *Alu* sequence as a probe. Experimental conditions were similar to those in Fig. 5. Lanes: 1 and 2, a recipient mouse TS$^-$ mutant and the transformant C0, respectively; lanes 3 through 10, clones 1 through 8, respectively, of spontaneous TS$^-$ mutants.

chromosome are stimulated when DNA DSBs are introduced into the donor marker gene (31). On the basis of this model, introduction of DNA DSBs and repair of the resulting double-strand gaps with homologous duplexes are suggested to lead to crossover of the flanking markers. In support of this model, yeast mutants of the *rad52* group that are defective in meiotic recombination and spontaneous and induced mitotic recombinations are defective in DNA DSB repair (26). Moreover, only mutants of this group are more sensitive to the lethal effect of thymidylate stress than the wild type (26). In this respect, it is noteworthy that caffeine sensitized mouse FM3A cells to the lethal effect of thymidylate stress and reduced the appearance of TS^- segregants (Fig. 3A), since caffeine is thought to inhibit postreplication repair of DNA damage (28). To explain the molecular basis of the induction of TS^- segregants by thymidylate stress, it is tempting to speculate along these lines of evidence, that the DNA DSBs induced by thymidylate stress triggered an intrachromosomal homologous recombination in the transferred human TS gene.

It is of interest that the above genetic events, as well as chromosome aberrations induced by thymidylate stress, were blocked by cycloheximide. Since the chromosome aberrations induced by thymidylate stress were seen only where DNA replication was ongoing (19) and cycloheximide is known to inhibit both initiation and elongation of DNA replication (20), the target of this drug can be DNA replication. An alternative explanation of the effect of cycloheximide on the thymidylate starved cells is equally possible. Since cycloheximide is an inhibitor of protein synthesis, it could inhibit synthesis or activation of a certain inducible enzyme(s) upon thymidylate stress which acts on DNA and eventually gives rise to DNA DSBs.

Finally, it should be emphasized that thymidylate stress is a naturally occurring stimulus in physiological conditions. Many phenomena similar to thymidylate stress are known to occur in vivo. As described above, heritable fragile sites are expressed by mild thymidylate stress. These sites are preferentially sensitive to thymidylate stress, and their mode of expression is similar to that of isochromatid breaks seen in thymidine-starved mouse TS^- mutant cells (19). Methotrexate-treated human lymphocytes show nonrandom chromosome breaks (nonheritable fragile sites) at specific sites (6). Recently, the possibility was suggested from familial analysis of patients that the fragile site at band q27 on the X chromosome in affected persons is a spot influencing meiotic recombination (38). In patients with megaloblastic anemia due to vitamin B_{12} or folate deficiency, which leads to thymidylate stress, unusual chromosome aberrations and cytogenetic alterations are observed (14). It is therefore probable that genetic and cytogenetic stability could be affected by the combination of both a peculiar type of DNA structure and certain deviations in the activity of thymidylate biosynthesis in mammalian cells as demonstrated in the present study.

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