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Unequal Homologous Recombination between Tandemly Arranged Sequences Stably Incorporated into Cultured Rat Cells

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Cultured rat cells deficient in endogenous thymidine kinase activity (*tk*) were stably transformed with a recombination-indicator DNA substrate constructed in vitro by rearrangement of the herpes simplex virus *tk* gene sequences into a partially redundant permutation of the functional gene. The recombination-indicator DNA did not express *tk*, but was designed to allow formation of a functional *tk* gene via homologous recombination. A clonal cell line (519) was isolated that harbored several permuted herpes simplex virus *tk* genes. 519 cells spontaneously produced progeny that survived in medium containing hypoxanthine, aminopterin, and thymidine. Acquisition of resistance to hypoxanthine, aminopterin, and thymidine was accompanied by the rearrangement of the defective *tk* gene to functional configuration. The rearrangement apparently occurred by unequal exchange between one permuted *tk* gene and a replicated copy of itself. Recombination was between 500-base-pair tracts of DNA sequence homology that were separated by 3.4 kilobases. Exchanges occurred spontaneously at a frequency of approximately 5×10^{-6} events per cell per generation. Recombination also mediated reversion to the tk^- phenotype; however, the predominant mechanism by which cells escaped death in the presence of drugs rendered toxic by thymidine kinase was not recombination, but rather inactivation of the intact *tk* gene.

Genomic evolution has long been thought to involve duplication-deletion events mediated by unequal homologous recombination between repeated DNA sequences (20, 23, 29, 42, 46). Recent evidence gathered through the analysis of cloned mammalian genes has validated the idea that unequal recombination is involved in DNA rearrangement in the germ line of mammals. Many thalassemia mutations are deletions incurred by unequal recombination between closely related members of the globin gene family (12, 23, 52). Duplications of globin genes, the expected reciprocal products of the unequal exchanges that generate thalassemias, have also been described (52). Unequal recombination in germ line DNA has also been implicated in deletion in other gene clusters (3, 11, 31), deletion of retrovirus proviruses (15, 17), generation of gene families (3, 6, 23, 31), and formation of genes that show internal sequence repetition (7, 11, 39, 44, 51).

Somatic cells might also be expected to sustain genomic deletions and duplications due to unequal homologous recombination. Recent reports from three laboratories indicate that unequal sister chromatid exchange is a mechanism by which genomic rearrangements occur in cultured mouse cells (25, 26, 41).

We now report the construction and characterization of a cultured rat cell line that allowed quantitation of the frequency of a homologous recombination event that appears to occur by unequal sister chromatid exchange. The cell line (519) contained as a stable insertion a permuted, partially redundant, defective thymidine kinase (*tk*) gene. 519 cells spontaneously produced progeny that survived in medium containing hypoxanthine, aminopterin, and thymidine (HAT). Acquisition of HAT resistance was accompanied by the rearrangement of the defective *tk* gene to functional configuration. The rearrangement appeared to occur by unequal exchange between the permuted *tk* gene and a replicated copy of itself. Recombination was between 500-base-pair tracts of DNA sequence homology that were separated by 3.4 kilobases (kb). Exchanges occurred spontaneously at a frequency of at least 5×10^{-6} events per cell per generation. Recombination also mediated reversion to the tk^- phenotype; however, the predominant mechanism by which cells escaped death in the presence of drugs rendered toxic by thymidine kinase was not recombination, but rather inactivation of the intact *tk* gene.

MATERIALS AND METHODS

Cell culture. Rat 3 cells, a gift from B. Topp, have been described previously (48). They were grown in Dulbeccos modified essential medium (DME; GIBCO Laboratories, Grand Island, N.Y.) with 10% fetal bovine serum (FBS, GIBCO). 519 cells were cultured in DME plus either 5% newborn bovine serum (NBS) or 5% FBS. Bromodeoxyuridine (BUdR) and thymidine arabinoside (araT) were used at 6.5×10^{-4} M. HAT medium contained DME plus 5% NBS and 1×10^{-4} M hypoxanthine, 4.5×10^{-7} M aminopterin, and 4.1×10^{-5} M thymidine. Cells were monitored for mycoplasmal contamination by the mycotect assay (Bethesda Research Laboratories, Inc., Bethesda, Md.) and the Hoechst stain procedure of Russell et al. (38). Cell cultures were incubated at 37°C unless otherwise indicated.

DNA construction. Plasmid p5.1 (Fig. 1) was constructed from DNA fragments derived from four sources: (i) pBR322(30), (ii) pTK2(8), (iii) simian virus 40 (SV40) strain TsA58 (21, 47), and (iv) bacteriophage lambda. Plasmid pTK2 is pBR322 bearing at its *Bam*HI site a 3.4-kb *Bam*HI fragment that encodes the thymidine kinase gene of herpes simplex virus (HSV) type 1. In pTK2, the 3.4-kb *Bam*HI fragment is oriented such that its single *Bgl*II site is proximal to the *Eco*RI site of the pBR322 vector molecule. The position of the *Bgl*II site within the 3.4-kb *Bam*HI fragment

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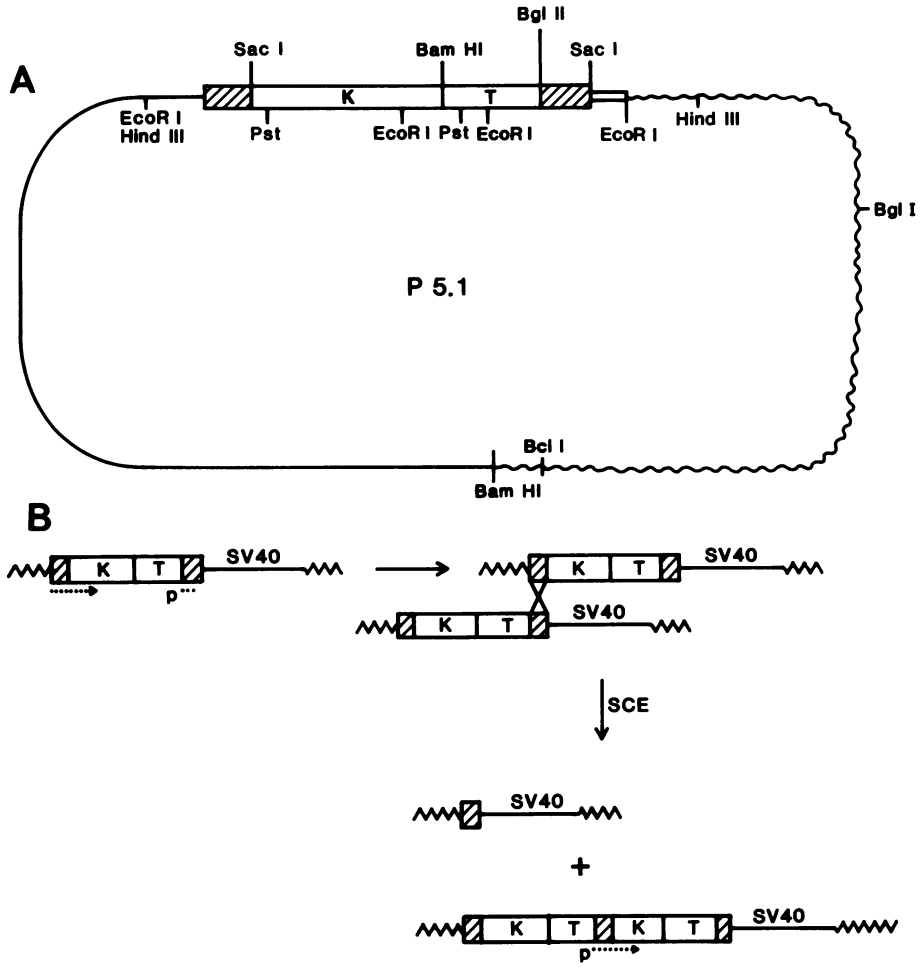

FIG. 1. Structure of the recombination-indicator DNA substrate and a possible mode of its rearrangement. (A) Plasmid p5.1 was constructed as described in Materials and Methods. Symbols: ▭, HSV DNA; ▨, tandemly repeated HSV DNA; ▭, DNA from phage λ; ∿∿, SV40 DNA; ——, pBR322 DNA. (B) An unequal homologous exchange mechanism for formation of an intact *tk* gene from two permuted units. Symbols: ▭, HSV DNA; ▨, repeated HSV DNA; ——, SV40 DNA; ∿∿, cell DNA; p, promoter; ----→, *tk* gene.

can be seen in Fig. 3C. Plasmid pTK2 was digested with *Bgl*II plus *Hin*dIII, and the large fragment was purified. This *Bgl*II-*Hin*dIII fragment was cloned by ligation to the small *Hin*dIII-*Bam*HI fragment of pBR322 (30), followed by transformation of *Escherichia coli* DH-1 (30) to ampicillin resistance. The resulting plasmid was designated p13. A second plasmid (p32) was constructed from four components: (i) the large *Sac*I-*Sal*I fragment of pTK2 (the position of the *Sac*I site in the 3.4-kb *Bam*HI fragment can be seen in Fig. 3C), (ii) a 221-base-pair *Sac*I-*Eco*RI fragment from lambda DNA, (iii) the large *Eco*RI fragment of SV40 strain TsA58, and (iv) the small *Bam*HI-*Sal*I fragment of pBR322. From plasmid p32 was prepared a *Bam*HI fragment that contained all of the *tk* and SV40 sequences. This *Bam*HI fragment was cloned into the *Bam*HI site of p13 DNA to produce p5.1.

DNA transformation. Monolayer cultures (80 cm^2) of rat 3 cells were transfected with 1 μg of p5.1 DNA plus 20 μg of rat carrier DNA by calcium precipitation (49). Parallel cultures of transfected cells were incubated at 32 or 39°C for 4 weeks in DME–5% FBS. The medium was changed every 5 days. Approximately 25 morphologically transformed foci appeared on each of the plates incubated at 32°C, but no foci occurred on the plates incubated at 39°C. This was the expected result, because the SV40 DNA in p5.1 encoded a temperature-sensitive large T antigen (21). Transformants were picked by teasing one lumped cell mass from each of five plates. Cells derived from transformed foci were propagated in DME–5% FBS at 37°C.

Preliminary characterization of morphological transformants. As a preliminary indication of the presence of p5.1 DNA, transformed cells were screened for the presence of SV40 T-antigen by indirect immunofluorescence as described previously (13). Cells (10^8) of each T-antigen-positive transformed focus were tested for the ability to survive growth in HAT. Culture dishes (80 cm^2) were seeded with 5×10^6 cells per dish in DME–5% NBS. At 24 h after plating, the culture medium was removed and replaced with HAT medium. HAT-resistant (HAT^r) colonies were scored 10 days after the addition of HAT. A cell line designated 519 produced approximately 25 HAT-resistant colonies per culture dish and was chosen for further study.

Isolation of HAT-resistant 519 clones. One 80-cm^2 culture dish of 519 cells was split 1:10 into 5% NBS–DME. After 4 days, the medium was aspirated from the 10 culture plates, and the cell monolayers were overlaid with 5% NBS–HAT. The medium was changed 48 h later and every fourth day thereafter. Unfixed HAT-resistant colonies were assessed visually after 13 days. One resistant colony was harvested

from each of five plates. Cells derived from HAT^r colonies were propagated in 5% NBS–HAT.

Extraction of cellular DNA. Culture medium was aspirated from 80-cm^2 monolayers of cells, and the cells were washed with phosphate-buffered saline. Cells were detergent lysed with 0.8 ml of Hirt buffer (10 mM Tris, [pH 7.5], 0.1 M NaCl, 20 mM EDTA, 0.2% sodium dodecyl sulfate. Pronase (50 μl of a 20-mg/ml solution, self-digested at 37°C for 1 h) was added to degrade cell nucleases. DNA extracts were transferred to tubes and incubated overnight at 37°C. Protein was removed by extraction with equal volumes of phenol and an isoamyl-chloroform mixture (1:24). Phenol was subsequently removed by two extractions with isoamyl-chloroform followed by three chloroform extractions. DNA was precipitated by the addition of 2 volumes of ice-cold ethanol to the aqueous phase. Precipitated DNA strands were removed from ethanol by spooling onto a bent Pasteur pipette. The DNA was suspended in a small volume (usually 0.3 ml) of double-distilled water. DNA concentrations were quantitated at 260 nm in a Gilford model 240 spectrophotometer.

Restriction endonuclease digestions. Restriction endonuclease digestions were performed with 6 μg of cell DNA in 30 μl. Reaction conditions were as recommended by the vendor (New England BioLabs, Inc., Beverly, Mass.). Digestions were conducted at 37°C for 2 to 3 h. Digested samples were loaded into wells of a 1% agarose gel and electrophoresed in Tris-phosphate buffer (40 mM Tris, 1 mM EDTA, 30 mM sodium phosphate, pH 7.6).

Blotting and hybridization. Electrophoresis was allowed to proceed 20 to 22 h at 2 to 3 V/cm. DNA fragments in the gel were denatured in 0.2 N NaOH–0.6 M NaCl. Gels were neutralized with 1.5 M NaCl–0.5 M Tris (pH 7.5). DNA was blot transferred by the method of Southern (43) in 10× SSC (1.5 M NaCl, 150 mM sodium citrate) overnight to nylon filters (Zeta probe, AMF-CUNO) as described in Maniatis et al. (30). Filters were prehybridized overnight at 65°C in sealed bags containing 5× SSPE (0.75 M NaCl, 50 mM sodium phosphate [pH 7.4], 5 mM EDTA), 5× Denhardt solution (0.1% Ficoll, 0.1% bovine serum albumin, 0.1% polyvinyl pyrrolidone), 0.4% sodium dodecyl sulfate, and 100 μg of denatured salmon sperm DNA per ml. Approximately 10 ml of this solution per blot (approximately 300 cm^2) was used. Blots were probed with nick-translated, ^{32}P-labeled DNA (30), approximately 5×10^7 to 7×10^7 cpm per bag, overnight at 65°C. Filters were washed with 0.1× SSC–0.1% sodium dodecyl sulfate at 60°C. Filters were exposed for autoradiography at −70°C in X-ray film cassettes containing two intensifying screens (Cronex extra life; Du Pont Co., Wilmington Del.).

Thymidine kinase assay. Thymidine kinase activity was assayed as described previously (34). Protein was determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.). Bovine serum albumin was used as a standard.

Genetic tests. For fluctuation analysis (28) 519 cells were plated in 5% FBS–DME at 10^4 cells per 3-cm^2 culture dish. A control set of 24 cultures was changed to HAT medium 17.5 h after plating. The remaining 31 cultures were allowed to grow for 6 days in 5% FBS–DME, after which time the cell number was determined by counting two trypsinized cultures with a hemocytometer. Two experiments were performed with the 31 cultures. In experiment I, 19 DME-grown cultures were changed to HAT in situ. In experiment II, 10 DME-grown cultures were trypsinized, suspended in HAT medium, and transferred to 10 75-cm^2 flasks, one culture per flask. The frequency of HAT resistance was estimated by the Poisson formula $P(0)\ e^{-a}$, where $P(0)$ is the probability that a plate of cells would not produce any HAT-resistant colonies and a is the average number of HAT-resistant cells per plate.

To assess the rate of recombination per cell generation, 519 cells were first cultured in medium containing araT for 7 days (three passages) to purge the cell population of cells expressing thymidine kinase. Cells grown in araT were used to seed two sets of 80-cm^2 culture plates. One set of cultures was seeded with 5×10^5 cells per plate and incubated in DME supplemented with 4×10^{-5} M thymidine and 10^{-4} M hypoxanthine (HTDME). The other set of cultures was seeded at 2×10^5 cells per plate and incubated in HAT. The HTDME cultures were allowed to grow for 72 h, at which time the cells were counted and used to establish secondary HTDME and HAT cultures. After 72 h of growth in HTDME, the secondary HTDME culture was used to seed a third set of HAT cultures. HAT^r colonies were counted after 14 days in HAT. The rate of emergence of HAT resistance was calculated as described previously (14).

RESULTS

Construction of a recombination-indicator cell line. Rat 3 cells, which are thymidine kinase deficient (*tk*$^-$) and contact inhibited, were transfected by calcium precipitation with p5.1 DNA, a molecule that contains two elements: (i) the early region of the SV40 genome and (ii) a recombination-indicator gene constructed from a HSV *tk* gene (Fig. 1A). In its input form, the recombination-indicator DNA cannot produce enzyme because the promoter is disconnected from the body of the gene. However, a functional *tk* gene can be derived from the input DNA via unequal homologous recombination (Fig. 1B). Plasmid p5.1 was designed to allow installation of the recombination-indicator gene in its input form via transfection followed by selection for morphological transformation caused by the SV40 moiety of p5.1. After transfection of rat 3 cells with p5.1, there appeared foci of morphologically transformed cells overgrowing the cell monolayer. These foci of overgrowth occurred at a frequency of about 1 per 10^6 transfected cells and contained SV40 large T-antigen protein as determined by indirect immunofluorescence (13). Analysis of primary transformants by Southern blotting of total cell DNA followed by hybridization to radioactive HSV *tk* DNA identified a clone, 519, that contained the recombination-indicator gene in its input form and lacked detectable intact *tk* DNA.

Data mapping the recombination-indicator insertion in 519 cells are shown in Fig. 2A. 519 cell DNA contained *Eco*RI, *Hin*dIII, *Pst*I, *Sac*I, and *Hin*dIII-*Bam*HI DNA fragments that comigrated with DNA fragments produced by digestion of p5.1 DNA. 519 cell DNA contained no detectable intact *tk* DNA. Intact *tk* genes would produce a 2.4-kb *Eco*RI fragment and a 3.4-kb *Bam*HI fragment. The positions at which such fragments would migrate are indicated in Fig. 2A. The results shown in Fig. 2A were obtained by hybridization of blotted DNA to a radioactive probe specific for the region designated K in p5.1 DNA (cross-hatched in Fig. 2B). Hybridization with other radioactive probes homologous to either the T sequences of p5.1 (Fig. 1A) or to pBR322 DNA produced results in accord with those shown in Fig. 2A.

HAT-resistant cells present in the 519 cell population contained an intact *tk* gene. When 519 cells were plated in HAT medium, resistant cells emerged at a proportion of about 5 per 10^6 cells plated. Analysis of the DNA in five HAT^r derivatives of 519 cells (5191c, 5192c, 5197c, 5198c, 5199c)

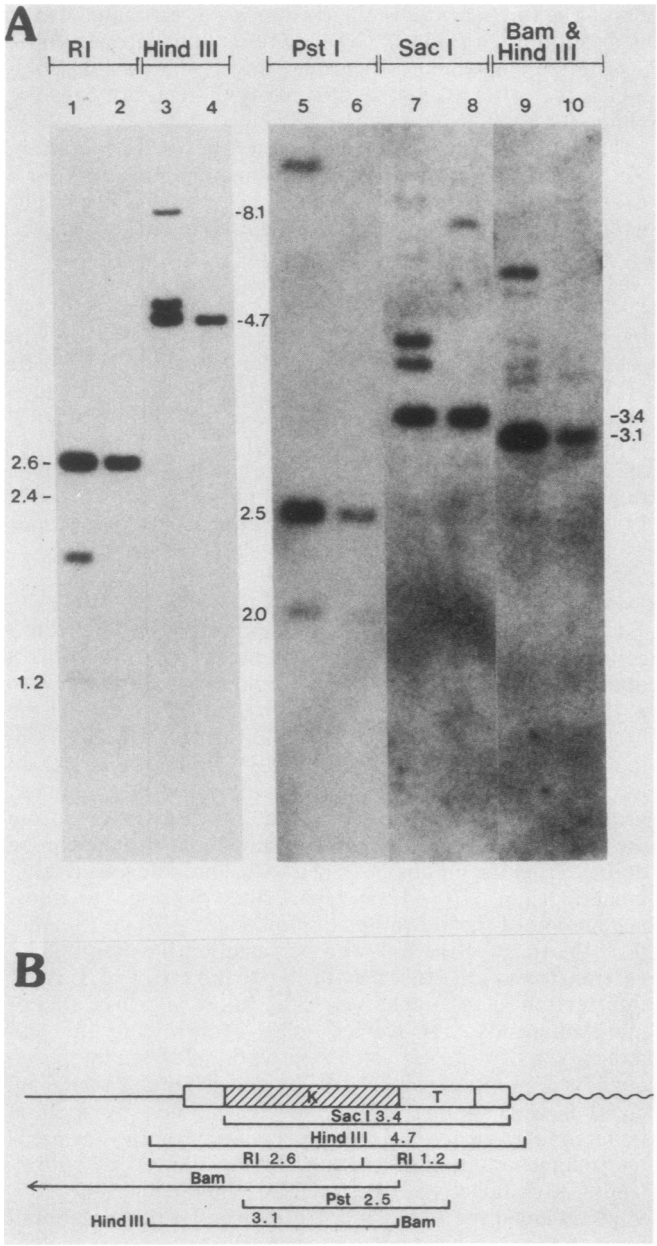

FIG. 2. Southern blot analysis of 519 cell DNA. (A) Blotted gels were hybridized to radioactive probe DNA corresponding to the cross-hatched region shown in panel B. Lanes: 1, 3, 5, 7, and 9, 519 cell DNA, 5 μg per lane; 2, 4, 6, 8, and 10, p5.1 DNA, 2×10^{-5} μg per lane. Numbers in the margins indicate fragment sizes in kilobase pairs. (B) restriction map of the HSV segment of p5.1 DNA. The numbers following each restriction enzyme abbreviation indicate the size of fragments in kilobase pairs. Symbols: ▭, HSV DNA; ∿∿, SV40 DNA; ——, pBR322 DNA.

showed that resistance to HAT was correlated with the acquisition of an intact HSV *tk* gene. More importantly, the data indicated that these *tk* genes were formed by homologous but unequal recombination between two copies of the same permuted *tk* insert. The presence in HAT^r 519 cells of DNA fragments that comigrated with those of an authentic HSV *tk* gene (pTK2) digested with either *Eco*RI or *Bam*HI is shown in Fig. 3A. HAT^r 519 clones also contained fragments that comigrated with those produced by digestion of the HSV *tk* gene with either *Pst*I or *Pvu*II (data not shown). HAT^r 519 clones also contained thymidine kinase enzyme activity. Specific thymidine kinase activities of cytoplasmic extracts of 519 cells and four HAT^r 519 clones (5191c, 5192c, 5198c, 5199c) are shown in Table 1. 519 cells possessed a small amount of thymidine kinase activity. This activity may be the result of expression of carboxy-terminal peptide from a secondary internal promoter known to be in the HSV *tk* gene (35). Whatever its origin, this minor activity was insufficient to allow growth in HAT.

The data indicating that the *tk* genes in HAT^r 519 cells were formed by unequal recombination between two copies of the same permuted *tk* insert are shown in Fig. 3B. Digestion of parental 519 cell DNA with *Bcl*I produced four bands that hybridized to radioactive HSV *tk* DNA: 30, 14, 13, and 9 kb (Fig. 3B, lanes 1 and 3). *Bcl*I patterns of DNA from five 519 HAT^r clones hybridized to K probe are shown in lane 2 and lanes 4 through 7 of Fig. 3B. All five HAT^r clones retained the 14- and 9-kb bands, but lacked the 13-kb band. Three of the five HAT^r clones (Fig. 3B, lanes 2, 4, and 6) also lost the 30-kb DNA present in *Bcl*I digests of 519 cell DNA. Two HAT^r clones, 5197c and 5199c (Fig. 3B, lanes 5 and 7), retained HSV *tk* DNA of high molecular weight. However, there appeared to be some loss of this material and a slight shift in migration compared with the 30-kb band in 519 cell DNA. We have not further characterized the changes in the 30-kb DNA of clones 5197c and 5199c, and we have not further examined the origin of the 10-kb band present in *Bcl*I-digested 5197c DNA. All five HAT^r clones contained a DNA fragment of 16 to 17 kb (designated 16.4 kb hereafter) that produced a radioactive signal stronger than that emitted from the 14- and 13-kb bands. The loss of the 13-kb band, the shift in molecular size, and the amplified *tk* signal all suggested that the 16.4-kb band was produced by unequal recombination involving two copies of the 13-kb *Bcl*I fragment of 519 DNA.

The idea that the 16.4-kb *Bcl*I band in HAT^r 519 clones was produced by unequal recombination between two copies of the 13-kb *Bcl*I fragment was further supported by the analysis of a HAT-sensitive revertant of HAT^r clone 5192c. The DNA of this revertant, 51922, contained three *Bcl*I bands with homology to the K probe (Fig. 4, lane 2). Two of these bands corresponded to the 14- and 9-kb bands present in 5192c and 519 DNAs (Fig. 4, lanes 3 and 1, respectively).

TABLE 1. Specific thymidine kinase activities in cell extracts

Cell line	Growth, in HAT	cpm in TMP[a]	Protein[b] (μg)	Thymidine, kinase, sp act[c]	Ratio-cell line/519	Presence of intact *tk* gene[d]
Rat 3	−	0	16	0		−
519	−	146	52	2.8	1.0	−
5191	+	3,272	41	79.8	28.5	+
5192	+	2,690	44	61.1	21.8	+
5198	+	2,045	41	49.9	17.8	+
5199	+	2,124	42	50.6	18.1	+
51922	−	25	28	0.9	0.3	−
51924	−	24	54	0.4	0.1	−
5192A4	−	70	45	1.5	0.5	−
5192A3	−	102	58	1.8	0.6	+
5192A9	−	19	20	1.0	0.4	+

[a] Determined by conversion of [^{14}C]thymidine to [^{14}C]TMP as described in Materials and Methods.
[b] Determined by the Bio-Rad protein quantitation procedure.
[c] Counts per minute per microgram of protein.
[d] Determined by Southern blot analysis.

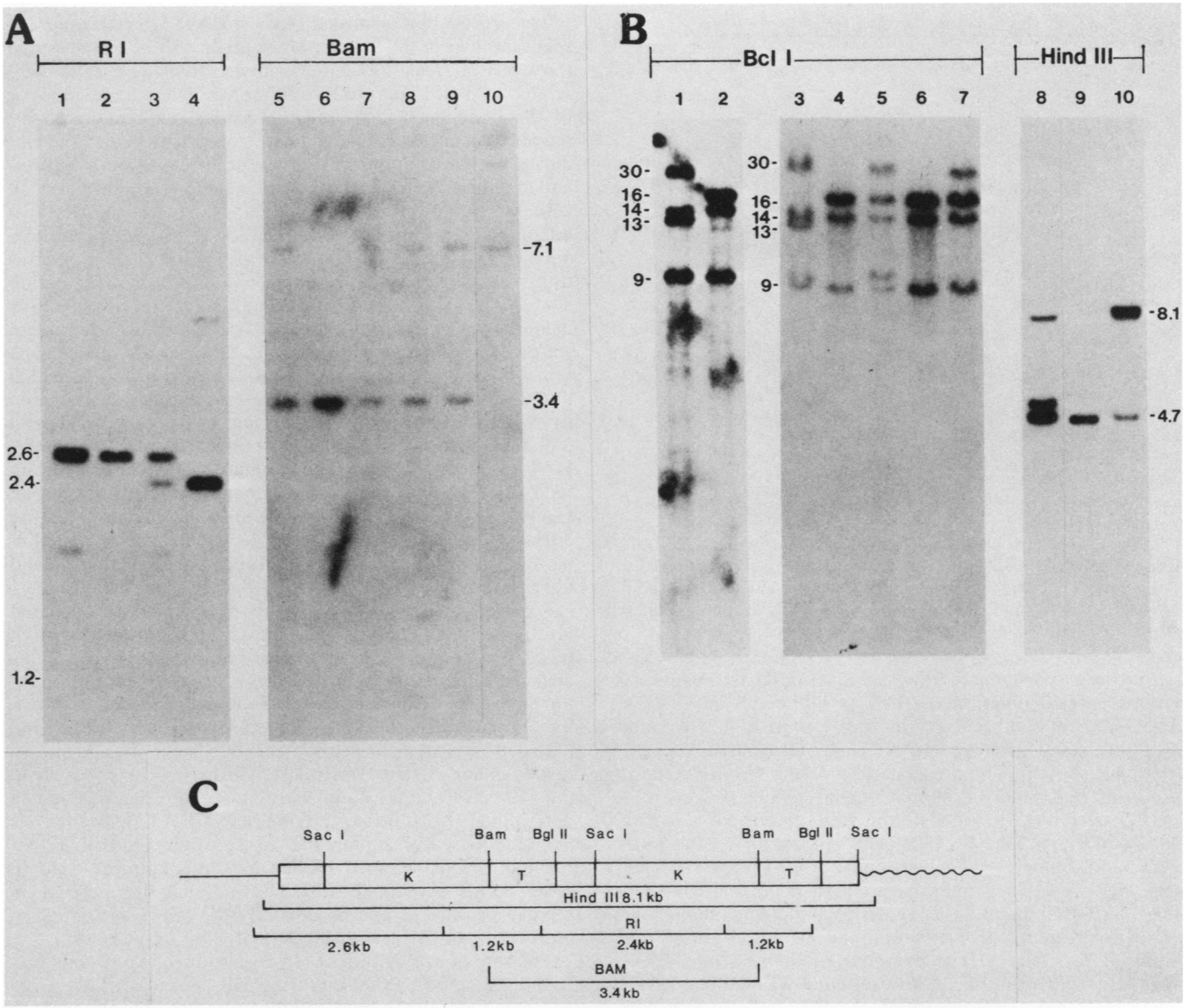

FIG. 3. Southern blot analysis of 519 cell DNA and DNA from HAT-resistant derivatives of 519 cells. Lanes containing cell DNAs were loaded with 5 μg of DNA per lane. Blots were hybridized to the K probe as designated in Fig. 2B. (A) Lanes: 1 and 10, 519; 2, p5.1; 4 and 6, intact *tk*; 3 and 5, 519 HAT^r clone 5192c; 7, HAT^r clone 5197c; 8, HAT^r clone 5198c; 9, HAT^r clone 5199c. (B) Lanes: 1, 3 and 8, 519; 2 and 10, 5192c; 4, 5191c; 5, 5197c; 6, 5198c; 7, 5199c; 9, p5.1. (C) Restriction map of the structures expected to be present in HAT^r 519 clones.

The third *Bcl*I band in 51922 DNA corresponded to the 13-kb band that had been lost from HAT^r 519 clones. 51922 DNA also lost the *Eco*RI and *Bam*HI *tk* fragments present in HAT^r 519 clones (data not shown), and extracts of 51922 cells lacked thymidine kinase activity (Table 1). The reappearance of a 13-kb *Bcl*I band in 51922 cells strongly supports the idea that the 16.4-kb *Bcl*I band in HAT^r 519 clones was formed from the 13-kb *Bcl*I band, and that this process is reversible.

The nature of the recombination event that produced the 16.4-kb *Bcl*I band was further elucidated by examination of *Hin*dIII digests of DNA from 519 cells, HAT^r 519 clones, and revertants of HAT^r clones. The DNA band pattern produced by *Hin*dIII digestion of DNA from 519 cells and from the HAT^r clone 5192c are shown in Fig. 3B, lanes 8 and 10, respectively. Hybridization of *Hin*dIII-digested 519 cell DNA to the K probe revealed three bands, which emitted radioactive signals of different intensity (Fig. 3B, lane 8). The band that produced the strongest signal comigrated with the 4.7-kb fragment produced by cleavage of p5.1 DNA with *Hin*dIII (Fig. 3B, lane 9). The identity of the DNA in the 4.7-kb band of 519 DNA was verified by double digestions with either *Hin*dIII plus *Bam*HI or *Hin*dIII plus *Bgl*II (data not shown). *Hin*dIII-digested DNA of HAT^r clone 5192c (Fig. 3B, lane 10) contained a new band that was approximately 3.4 kb larger than the 4.7-kb *Hin*dIII band in 519 cell DNA (lane 8). This is the expected result if formation of an intact *tk* gene were mediated by unequal recombination between two 4.7-kb *Hin*dIII fragments. Another prediction of such a model is that the 4.7-kb *Hin*dIII band should be diminished in the DNA of HAT^r 519 clones. The degree of diminution in the signal generated from the 4.7-kb band would depend on how many 4.7-kb *Hin*dIII fragments resided in 519 cell DNA. The 4.7-kb *Hin*dIII band did not disappear from 5192c DNA, but the signal was greatly reduced.

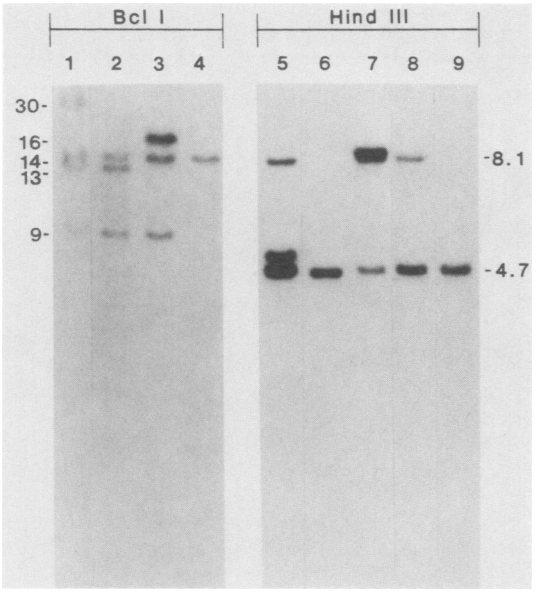

FIG. 4. Southern blot analysis of tk^- revertants of 5192c cells. Cell DNAs were loaded at 5 μg per lane. Blots were hybridized to the K probe as designated in Fig. 2B. Lanes: 1 and 5, 519; 3 and 7, 5192c; 6, p5.1; 2 and 8, 51922; 4 and 9, 51924. 51922 and 51924 are BUdR-resistant clones derived from 5192c cells.

Further experiments showed that *Hin*dIII fragments 4.7 kb in size resided in three of the four *Bcl*I bands in 519 cells. The 9-kb *Bcl*I band failed to hybridize to pBR322 DNA and therefore could not have harbored a 4.7-kb *Hin*dIII fragment (data not shown). The presence of a 4.7-kb *Hin*dIII band in the 14-kb *Bcl*I band was shown by analysis of a tk^- revertant of 5192c cells (51924 cells), the *Bcl*I pattern of which is shown in Fig. 4, lane 4. 51924 cells had lost all but the 14-kb *Bcl*I band. Since 51924 cells retained a 4.7-kb *Hin*dIII band (Fig. 4, lane 9), there must be such an *Hin*dIII fragment in the 14-kb *Bcl*I band. The evidence indicating that 4.7-kb *Hin*dIII fragments were present in the 30- and 13-kb 519 *Bcl*I bands is less direct. The presence of at least one 4.7-kb *Hin*dIII fragment in the 30-kb *Bcl*I band was implied by the analysis of the 51922 revertant of 5192c cells, which contained *Bcl*I bands 14, 13, and 9 kb in size. (Fig. 4, lane 2). The *Hin*dIII pattern of 51922 DNA is shown in lane 8 of Fig. 4. The radioactive signal emitted from the 4.7-kb *Hin*dIII band of 51922 DNA was half that emitted from the 4.7-kb *Hin*dIII band of 519 cells (Fig. 4, lane 5). This loss of signal was concomitant with the loss of the 30-kb *Bcl*I band from 51922 cells (by way of 5192c cells), suggesting that one or more 4.7-kb *Hin*dIII fragments were present in the 30-kb *Bcl*I band of 519 cell DNA. Analysis of 51922 cell DNA also indicated that the 13-kb *Bcl*I band of 519 cell DNA harbored a 4.7-kb *Hin*dIII fragment. This conclusion was indicated by the correlation between the regeneration of the 13-kb *Bcl*I band (Fig. 4, lane 2) and the increase in the signal emitted from the 4.7-kb *Hin*dIII fragment of 51922 cell DNA as compared with the signal emitted from the 4.7-kb *Hin*dIII band in 5192c cell DNA (compare lanes 7 and 8 of Fig. 4). 51922 cell DNA (Fig. 4, lane 8) retained an *Hin*dIII band that migrated slightly faster than the 8.1-kb band in 519c DNA (lane 7). This 8.0-kb DNA is the same as that in 519 DNA (Fig. 4, lane 5) which contained no intact *tk* gene (Fig. 1, lanes 1 and 9). Therefore, the 8.0-kb material is distinct from the 8.1-kb DNA, which harbored the intact *tk* gene in 5192c cells.

The process that formed *tk* genes in HAT^r 519 cells occurred spontaneously in the absence of selection. Physical analysis of the DNA of HAT^r 519 clones showed that these cells were related to the 519 parent, providing strong evidence in favor of the conclusion that intact *tk* genes were acquired by recombination after establishment of the 519 cell line, not during the transfection step. To corroborate this conclusion, and to determine the rate at which *tk* genes were formed, 519 cells were subjected to two kinds of genetic analysis: (i) fluctuation tests and (ii) determination of the rate of appearance of HAT^r cells as a function of the number of generations of cell growth in nonselective medium. Fluctuation analysis was performed by plating 519 cells in nonselective medium in 55 culture dishes at 10^4 cells per dish. A control set of 24 dishes was cultured in HAT from the outset. The remaining cultures were allowed to grow for 7 days, at which time the 31 plates were divided into two experimental sets. In experiment I, a set of 19 plates was switched to HAT, and 2 plates were trypsinized for determination of cell number. HAT-resistant colonies were counted 10 days after addition of HAT. The results of this experiment (Table 2) showed that HAT resistance arose spontaneously in the 519 culture during growth in the absence of HAT. The rate of recombination was calculated from the proportion of plates that did not produce any HAT^r clones by the Poisson formula (27).

Experiment II in this analysis (Table 2) showed that HAT-resistant cells arose at random times during growth in the absence of selection. In experiment II, 10 cultures of 519 cells were treated as described above, but instead of switching the established monolayers of cells that had been grown for 7 days directly to HAT, the cultures were trypsinized and plated in HAT at a density of 6×10^3 cells per cm^2. Again the results indicated that HAT resistance arose spontaneously in the 519 culture at a rate in accordance with that previously determined. In addition, the number of HAT-resistant cells fluctuated greatly among the 10 cultures, indicating that recombination occurred earlier in some cultures than in others. This experiment also showed that the increase in number of cultures that produced HAT^r cells was not due to increased cell density at the time of addition of HAT.

The rate of acquisition of HAT resistance in 519 cells was also determined by first culturing 519 cells in araT to purge tk^+ cells from the culture, followed by growing the cells in the absence of araT for three and six generations, at which time the cells were plated in HAT (Table 3). HAT^r cells accumulated after removal of araT at a rate comparable to that determined by fluctuation analysis (Table 2).

TABLE 2. Spontaneous emergence of HAT-resistant cells from 519 populations grown in the absence of selection[a]

Expt	No. of cells seeded per plate	No. of cells per plate at time of addition of HAT	Cell density at time of addition of HAT (cells/cm^2)	No. of plates with HAT^r colonies/ total	Avg no. of events per cell[a]
Control	10^4	10^4	3×10^3	2/24	8.7×10^{-6}
I	10^4	2.7×10^5	9×10^4	16/19	6.8×10^{-6}
II	10^4	4.3×10^5	6×10^3	5/10[b]	1.6×10^{-6}

[a] Calculated from the probability that a plate would contain no HAT^r cells (*Po*) by the formula $Po = e^{-a}$, where a equals the average number of events per plate.

[b] The fluctuation of HAT resistance among 10 separate cultures was 0, 2, 11, and 40 HAT^r colonies from 5, 3, 1, and 1 culture, respectively.

TABLE 3. Emergence of HAT^r cells from 519 populations that had been purged of tk^+ cells by exposure to araT

No. of generations of growth in DME after treatment with araT	No. of HAT^r clones per 10^6 cells tested	Mutation rate[a]
0	0	
2.78	6	3.0×10^{-6}
6.08	16	3.6×10^{-6}

[a] Calculated from the formula $m = 2 \ln 2(M/Ng)$, where m is the mutation rate, M is the number of HAT^r cells, N is the number of cells, and g is the number of generations (see reference 14). Results are given as cells per generation.

HAT-resistant 519 cells lose *tk* function via unequal recombination, deletion, and repression. Revertants of 5192c cells were obtained by plating in medium containing either BUdR or araT. Both these analogs of thymidine kill tk^+ cells. Fluctuation analysis showed that cells resistant to either BUdR or araT arose spontaneously in the absence of selection at a rate of approximately 4×10^{-3} per cell generation (data not shown).

Twenty putative tk^- clones, 10 from araT cultures and 10 from BUdR cultures, were grown under selective conditions. DNA was harvested and analyzed by Southern blotting as described above. The data indicated that two clones, one selected in BUdR and one selected in araT, had lost the *tk* gene and had regained the permuted *tk* structure originally installed in 519 cells. Analysis of one such clone, 51922, is shown in Fig. 4. Surprisingly, recombinants such as 51922 accounted for only 10% of the resistance to thymidine analogs; 70% of the resistant clones retained the *tk* gene, whereas 20% deleted it entirely. The mechanism by which *tk* activity was lost from cells retaining the intact gene is unclear at this time. Lysates of araT-resistant cells that contained an apparently intact *tk* gene lacked thymidine kinase activity (Table 1), suggesting that the *tk* gene was either repressed or mutated in these cells. The frequency of araT resistance was extremely high at 4×10^{-3} per cell generation, suggesting modification as the mechanism of gene repression. Preliminary experiments indicate that the *tk* gene in araT-resistant clones is methylated (Meade and Stringer, unpublished data). Whatever the mechanism by which thymidine kinase function was lost, it could be recovered, albeit at a 1,000-fold lesser rate than the inactivation event. To determine the rate of recovery to tk^+, araT-resistant derivatives of 5192c cells were batch cultured in DME and then switched to HAT. HAT-resistant cells emerged at a rate of 5×10^{-6} per cell generation. Southern analysis of six of these second-generation HAT^r clones showed them all to contain the same *Bcl*I fragment pattern as 5192c cells (data not shown).

Our initial intent in selecting revertants of 5192c cells was to acquire a second point on a curve relating recombination frequency to target size. HAT^r 519 derivatives were the result of unequal recombination between 500-base-pair repeats. We expected that the *tk* gene in 5192c cells would also undergo unequal recombination and in so doing reform the permuted structure present in 519 cells. Such recombination events could be mediated by 3.9 kb of homology. Therefore if the frequency of recombination were linearly related to target size, one would expect the *tk* DNA in 5192c cells to rearrange to an inactive form approximately 8 times more often than HAT^r cells emerge from the 519 population, i.e., at a rate of 4×10^{-5} per cell generation. However, the rate at which araT-resistant cells were generated by unequal recombination exceeded the expected value by 10-fold. This could mean that the recombination rate may increase disproportionate to target size. Alternatively, regeneration of the 13-kb *Bcl*I fragment could occur via intramolecular excision, which may be more frequent than the intermolecular unequal exchanges that presumably mediate the loss of the 13-kb *Bcl*I fragment.

One BUdR-resistant clone of 5192c cells (51924) was found to have retained only the 14-kb *Bcl*I fragment (Fig. 4, lane 4). Further analysis of the DNA from 51924 cells showed the 14-kb *Bcl*I fragment to contain an *Hin*dIII fragment (Fig. 4, lane 9), a *Bam*HI-*Hin*dIII fragment, and a *Bgl*II-*Hin*dIII fragment that comigrated with those of p5.1 DNA (data not shown). Therefore, the 14-kb *Bcl*I fragment in 519 cells appeared to contain the same permuted *tk* gene that resides in the 13-kb *Bcl*I fragment. The 14-kb *Bcl*I fragment of 519 cells has not been observed to produce an intact *tk* gene. We do not yet know whether this is because the *tk* sequence in the 14-kb fragment is defective or repressed or because the 14-kb fragment does not recombine as frequently as the 13-kb fragment. The first and last alternatives engender interesting possibilities for future experiments, as discussed below.

DISCUSSION

Formation of the *tk* gene in 519 cells was accompanied by the loss of a 13-kb *Bcl*I DNA fragment and the acquisition of a new DNA fragment that was 3.4 kb larger than the parental 13-kb fragment. This 16.4-kb fragment appeared to contain two copies of HSV *tk* DNA. These observations can be most simply explained as the manifestation of unequal sister chromatid exchange between an integrated test gene and a copy of itself produced during chromosomal replication. Such a process is depicted diagramatically in Fig. 1B. It is likely that the permuted *tk* gene is integrated into a rat chromosome, because 519 cells have maintained the test gene DNA during prolonged culture in nonselective medium. Although deletion of the rearranged test gene did occur in tk^- revertants of HAT-resistant 519 cells selected by culture in araT or BUdR, the rate of deletion was comparable to the rate at which the *tk* gene was rearranged to the permuted form. This is consistent with a recombination mechanism of deletion rather than loss due to lack of covalent linkage to a chromosome.

Although unequal sister chromatid exchange is the most likely explanation, other mechanisms are possible. One alternative, excision-reintegration, seems highly unlikely because such a mechanism would require excision of the permuted *tk* gene resident in the 13-kb *Bcl*I DNA fragment, followed by site-specific reintegration into a second 13-kb *Bcl*I DNA fragment. Other alternatives to unequal sister chromatid exchange would be possible if the permuted *tk* gene in 519 cells were not integrated into a normal chromosome or if the structure that harbored the permuted *tk* gene, chromosomal or not, were not haploid. The permuted *tk* gene could be maintained in an unintegrated, episomal structure composed of input plasmid and rat carrier DNA. If this rather unlikely circumstance were to pertain, formation of an intact *tk* gene could either entail recombination of a haploid permuted test gene with a replicated copy of itself or recombination between two episomes in a cell that carried two or more such molecules. These possibilities seem unlikely, but cannot be ruled out at this time.

It is interesting to consider the implications of the idea that the rearrangements that rendered 519 cells resistant to HAT

were mediated by unequal sister chromatid exchanges. The frequency of rearrangement of the 519 test gene is nearly identical to the frequency at which an endogenous murine retrovirus has been found to depart the dilute locus (17). This correlation suggests that the frequency of unequal sister chromatid exchange as measured in 519 cells is of a biologically relevant magnitude, and that germ line and somatic cells may be similar in this aspect of genomic plasticity. The frequency of unequal homologous recombination in 519 cells is also very similar to that observed in mouse cells bearing various recombination-indicator substrates (25, 26, 41).

Comparison of the data derived from 519 cells with similar data on unequal sister chromatid exchange in vegetative *Saccharomyces cerevisiae* cells suggests that the genomes of mammals and *S. cerevisiae* may be similar in the rate at which they undergo mitotic unequal sister chromatid exchange. Szostak and Wu determined the probability of unequal recombination between two 9-kb rRNA genes to be 5×10^{-4} per cell generation (45). The 519 test gene underwent unequal recombination at a frequency of 5×10^{-6} per cell generation, but the recombination target was 18-fold smaller than the *S. cerevisiae* rRNA repeat unit. If the recombination rates in question are normalized for target size, the rates of mitotic unequal sister chromatid exchange in *S. cerevisiae* and rat cells differ only by a factor of 5. This is in contrast to the 400-fold difference in rates of meiotic recombination exhibited by *S. cerevisiae* and mouse cells. These findings suggest that the DNA in somatic mammalian cells is not vastly less likely to recombine than the DNA in mitotic *S. cerevisiae* cells. An additional consideration worth mentioning in this regard is that the frequency of homologous recombination as assessed in this and other studies (25, 26, 41) may be an underestimate due to the influence of DNA modification systems such as methylation, which could render some recombination events cryptic. Our analysis of reversion of 5192c cells to the tk^- phenotype indicated that inactivation of intact *tk* genes occurred at a rate of approximately 4×10^{-3} per cell generation. At this rate, 12% of the cells in a population of 519 cells that had been through 30 generations of growth (the number of generations required to produce 10^9 cells) would be expected to have lost the ability to express recombined genes.

Mammalian genomes contain hundreds of thousands of copies of interspersed, repeated elements that could mediate unequal recombination (16). If all such repeats were to behave like those that flank the permuted *tk* gene of 519 cells, every cell would experience a duplication-deletion event at nearly every division. However, a number of factors probably act to limit unequal recombination between repeated sequences. First, the homology between members of moderately repeated sequence families is not perfect, but averages around 90% (10, 36). Although the effect of sequence mismatch on the rate of recombination in mammalian cells is not known, in *Escherichia coli* recombination rates have been shown to drop almost 100-fold for 10% divergence between potential exchange participants (H. V. Huang, personal communication). Another factor that may act to limit the occurrence of unequal recombination is the smaller size of cellular repeats compared with the repeats that flank the permuted 519 test gene. At 500 base pairs, the repeats in the 519 test gene are three times larger than the BI interspersed repetitive element (18). Since recombination in mammalian somatic cells seems to be dependent on the length of homologous sequence available (37), this factor could reduce the rate of unequal recombination another threefold. Other factors such as the spacings of repeats and chromosomal position effects probably influence the rate of unequal recombination. Studies on recombination between *S. cerevisiae* rRNA genes have suggested that proximity positively influences the probability of unequal recombination (45). The repeats in the 519 test gene are closer together than most interspersed repeats in mammalian genomes (40). With regard to chromosomal position effects, we cannot rule out the possibility that the 519 test gene insertion may reside in an unusual structural milieu that may not be representative of the average chromosomal locus. Studies on recombination of recombination-indicator insertions in mouse cells (25, 26, 41) are subject to the same uncertainty.

Chromosomal position effects aside, it would not be unreasonable to expect deletion due to mitotic unequal recombination between members of an interspersed repeated sequence family to be 100-fold less likely to occur than formation of an intact *tk* gene in 519 cells. Although this rate would still be rather high in terms of deletions suffered per cell generation, such deletions would not be expected to frequently mediate the total loss of functions encoded by diploid genes, because such losses would require two hits at the same target. It should be noted that duplication deletion events mediated by four-stranded meiotic unequal homologous recombination between two interspersed repetitive elements such as Alu family sequences in humans has not been observed. The one case in which a deletion was incurred via Alu-Alu interaction appears to have been due to either nonhomologous recombination or to an intrastrand snap-back mechanism (24). By contrast, meiotic unequal sister chromatid exchange mediated by repeated genes of near-perfect homology is common (3, 12, 23, 31, 52).

519 cells should be a good model system in which to study the factors that control general homologous recombination in cells that have not been perturbed by DNA transfection, microinjection, or virus infection. Although it is well known that DNA delivered to cells by these routes recombines extremely well (1, 2, 4, 9, 19, 50), it is not clear whether this activity reflects the basal function of the recombination pathway, or whether introduction of foreign DNA induces recombination enzymes. 519 cells will be useful in addressing this question.

A number of chemical agents stimulate sister chromatid exchange at doses far below those required to induce detectable mutations or chromosomal aberrations. Therefore, sister chromatid exchange rates have been regarded as the most sensitive indicator of potential health hazards posed by low-level exposure to mutagenic substances (5, 22, 33). The mutational impact of increased sister chromatid exchange can now be directly examined by determining the relationship between equal sister chromatid exchange, which is a null event genetically, and unequal sister chromatid exchange, which causes deletions and duplications of DNA. Sister chromatid exchange has also been reported to increase in cells expressing the SV40 large T antigen (32). 519 cells contain SV40 DNA that encodes a temperature-sensitive large T antigen (21). All of the experiments described in this communication were conducted at 37°C, at which temperature the viral protein should be highly incapacitated if not totally inactive. However, we cannot rule out some contributions of the SV40 large T antigen to the rate of recombination seen in 519 cells. It should be interesting to see whether the frequency of recombination is 519 cells is temperature dependent.

The presence of multiple recombination test genes in the 519 cell line presents both problems and opportunities. One problem inherent in multicopy cell lines is that recombina-

tion could occur via exchange between different insertions as well as via unequal exchange between replicated copies of the same insertion. In this regard, data from multicopy lines are more difficult to interpret, and determination of the mechanism requires extensive physical analysis of the DNA sequences involved. On the other hand, the presence of more than one copy of a recombination test gene may be useful in the analysis of substrate influences on homologous recombination. In this regard, the data derived from the analysis of 519 cells are potentially quite interesting. 519 cells contained at least three *Bcl*I fragments that each contained a full copy of the permuted *tk* gene (Fig. 2). However, only the 13-kb fragment was observed to recombine. This could mean that the permuted *tk* gene in the other *Bcl*I fragments recombines less frequently than the test-gene in the 13-kb fragment. Alternatively, the test gene in the other *Bcl*I fragments might be mutated or modified and incapable of bestowing HAT resistance after recombination. It should be possible to determine the competency of the permuted *tk* gene that resides in the 14-kb *Bcl*I fragment by analysis of the 519-derived cell line that contains only the 14-kb *Bcl*I fragment (Fig. 4). If the test gene is not defective, the relatively infrequent recombination of the 14-kb *Bcl*I fragment may reflect a position effect on recombination frequency. If the test gene residing in the 14-kb fragment is defective, the cell line carrying it would be a good recipient for exogenous *tk* DNA fragments that could rescue HAT resistance via site-specific homologus recombination between the integrated and exogenous *tk* DNA fragments. Such a system would be useful in exploring the basis of the inefficiency of targeted gene replacement in cultured mammalian cells.

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