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Genetics

DNA-mediated gene transfer of a circular plasmid into murine cells

(thymidine kinase cotransfer/Southern blot hybridization/gene propagation)

KENNETH M. HUTTNER*, GEORGE A. SCANGOS†, AND FRANK H. RUDDLE*†‡

Departments of *Human Genetics and †Biology, Yale University, New Haven, Connecticut 06520

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ABSTRACT **We have used DNA-mediated gene transfer to introduce a recombinant plasmid containing the human β-globin gene (H_β 1) into cells of a mouse tissue culture line, Ltk⁻. DNA isolated from independent transfer lines was analyzed by restriction endonuclease digestion, gel electrophoresis, modified Southern blotting, and filter hybridization using H_β 1 as a probe. H_β 1 sequences were present in 80% of the lines at 1–30 copies per cell. Many of the lines gave a hybridization pattern indicative of H_β 1 sequences integrated into high molecular weight DNA. DNA from three cell lines, digested with several restriction enzymes, produced a pattern providing evidence for the presence of circular H_β 1 molecules in the murine recipient cells.**

The transfer of genetic material between mammalian tissue culture cells has its origin in the technique of whole cell fusion. Technical advances have allowed somatic cell geneticists to transfer subsets of a cell's chromosomal complement (microcells) (1), portions of individual chromosomes (chromosome-mediated gene transfer) (2, 3), and individual genes (DNA-mediated gene transfer) (4–6) into recipient cells. After the demonstration that the herpes simplex virus (HSV) thymidine kinase (TK) (EC 2.7.1.21) gene could be transferred into TK-deficient cells by calcium phosphate precipitation (transformation) (5), Wigler *et al.* (7) discovered that mammalian genes also could be used to transform recipient cells by this technique. In a recent report (8), Wigler *et al.* described a technique for the introduction of any known sequence into cultured cells. Cotransformation was accomplished by mixing the DNA containing a selectable marker—e.g., TK—with characterized DNA molecules—e.g., linear φX174. Such a mixture was used to transform Ltk⁻ cells to the TK⁺ phenotype. Cells capable of taking up and expressing the TK gene took up additional DNA. In an initial mixture with φX174 sequences present in up to 20,000-fold excess compared to TK gene copy number, over 90% of independently derived transformed lines maintained the nonselected sequences.

Wigler *et al.* reported on cotransformation with HSV TK and well defined, nonselected, *linear* sequences (8). We cotransformed Ltk⁻ cells with TK genes from several sources and nonselected *circular* plasmid molecules. Analysis of the state of the plasmid sequences in the recipient cells suggests that we have introduced a circular molecule which is capable of existing in a non-integrated form in murine cells.

MATERIALS AND METHODS

Cell Culture. The TK⁻ cell line, Ltk⁻, was a gift of S. Silverstein and was maintained in monolayer culture at 37°C in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum (Flow Labs, McLean, VA). All transfer experiments were performed in this medium containing 10% fetal bovine serum. Transfer lines were grown in alpha-minimal essential medium (GIBCO) containing 5% fetal bovine serum for subcloning experiments. Cell lines were tested periodically for mycoplasma contamination as described by Barile *et al.* (9).

DNA Sources. The plasmid H_β 1 (F.1) was a gift of T. Maniatis to B. G. Forget. This plasmid was constructed by inserting a 4.4-kilobase (kb) fragment of human DNA that contains the human β-globin gene, into the unique *Pst* I site of plasmid pBR322. HSV type 1 TK gene (HSV-1 TK) was isolated from intact HSV-1 DNA (10). High molecular weight DNA was isolated from HeLa cells (human) and CH-Rel.22cA4 cells (Chinese hamster) as described by Pellicer *et al.* (11). For blotting analysis DNA was isolated as described by Wigler *et al.* (8).

DNA-Mediated Gene Transfer. DNA-mediated gene transfer of TK into Ltk⁻ cells was accomplished by calcium phosphate precipitation as described by Wigler *et al.* (7) with the following modifications: (*i*) we used 10–30 μg of high molecular weight DNA per 10^6 cells for transfer of the cellular TK gene; (*ii*) the $CaCl_2$ solution was prepared in 10 mM Tris·HCl, pH 7.12; and (*iii*) hypoxanthine/aminopterin/thymidine (HAT) selection (12) was initiated at 30 hr post-DNA treatment. In our cotransformation experiments we mixed 1 μg of H_β 1 DNA either with 10 ng of HSV TK gene plus 20 μg of salmon sperm DNA or with 10–30 μg of cellular DNA per 10^6 recipient cells.

TK⁺ transformants were identified by growth in Dulbecco's medium/10% fetal bovine serum/HAT, and individual colonies were isolated and expanded for further analysis.

Electrophoresis and Filter Hybridization. The restriction endonucleases *Hin*dIII, *Xba* I, and *Kpn* I were purchased from New England Biolabs; *Eco*RI was prepared according to the method of Greene *et al.* (13). DNA from individual transformants was digested by using assay conditions suggested by New England Biolabs. A sample consisting of 10–15 μg of cellular DNA was electrophoresed on horizontal 0.7% or 1.0% agarose gels (Sigma) in 36 mM Tris·HCl/30 mM NaH_2PO_4/1 mM EDTA at pH 7.7 (8). Generally, the gels ran for 8–12 hr at 2–3 V/cm until marker dye had migrated 10–13 cm. Because cleavage of mouse cellular DNA with *Kpn* I or *Xba* I produced larger fragments than did *Eco*RI digestion, we electrophoresed these digests on 0.7% agarose gels at 2 V/cm for a longer time in order to maximize resolution of high molecular weight fragments. By using restriction digests of H_β 1 as controls, we

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Abbreviations: TK, thymidine kinase; kb, kilobase; HSV, herpes simplex virus; HSV-1, HSV type 1; HAT, hypoxanthine/aminopterin/thymidine.

‡ To whom correspondence should be addressed at Yale University, Kline Biology Tower, New Haven, CT 06520.

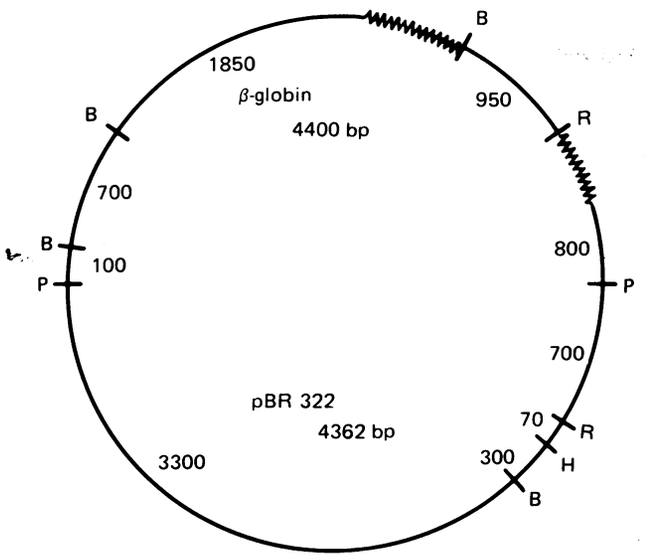

FIG. 1. Restriction map of H_β 1. This plasmid is a derivative of phage HβG 1 (17). Distances between restriction sites are indicated in number of base pairs. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst* I. *Kpn* I and *Xba* I do not cleave H_β 1. These restriction sites are in agreement with published results. ⁓, Globin coding sequences.

saw repeatedly that H_β 1 DNA run by itself migrated further than H_β 1 DNA electrophoresed in a background of digested cellular DNA. All of our controls consisted of H_β 1 DNA mixed with Ltk$^-$ DNA prior to digestion and electrophoresis.

The gels were treated consecutively with acid and alkali and neutralized as described by Wahl *et al.* (14). This treatment cleaves large DNA molecules in the gel into 1- to 2-kb fragments, which results in increased efficiency of transfer during blotting. The DNA was transferred to nitrocellulose filters (Schleicher and Schuell) (15) and baked at 80°C for 2–5 hr under reduced pressure.

H_β 1 DNA was labeled by nick-translation using ^{32}P-labeled nucleotide triphosphates (New England Nuclear) as described by Maniatis *et al.* (16) with minor modifications. The specific activity was 1–3 × 10^8 dpm/μg of DNA (1 dpm = 16.7 mBq).

Filter hybridization was performed by the procedure of Wahl *et al.* (14) by using a 3- to 4-hr prehybridization, an 8-hr hybridization, and two sets of posthybridization washes, one in 2 times concentrated standard saline citrate/0.1% sodium dodecyl sulfate and one in 0.1 concentrated standard saline citrate/0.1% sodium dodecyl sulfate. We used twice the recommended concentration of Denhardt's solution in all solutions. The posthybridization low-salt wash was extended to 30 min (done twice) at 65°C to decrease the background.

The filters were exposed to X-Omat x-ray film (Kodak) for 2–72 hr at −70°C using Cronex intensifying screens (Dupont).

Table 1. DNA-mediated gene transfer with Ltk$^-$ as recipient

Source of DNA	No. of colonies	No. of positive flasks	Colonies per 30 μg donor DNA/10^6 cells
Chinese hamster + H_β 1	6	2/4	1.5
Chinese hamster + H_β 1	25	10/10	7.5
Human (HeLa) + H_β 1	10	7/10	1.0
Salmon sperm + HSV-TK H_β 1	11	7/10	NA

Data indicate TK transformation frequency only. NA, not applicable to gene transfer with carrier DNA.

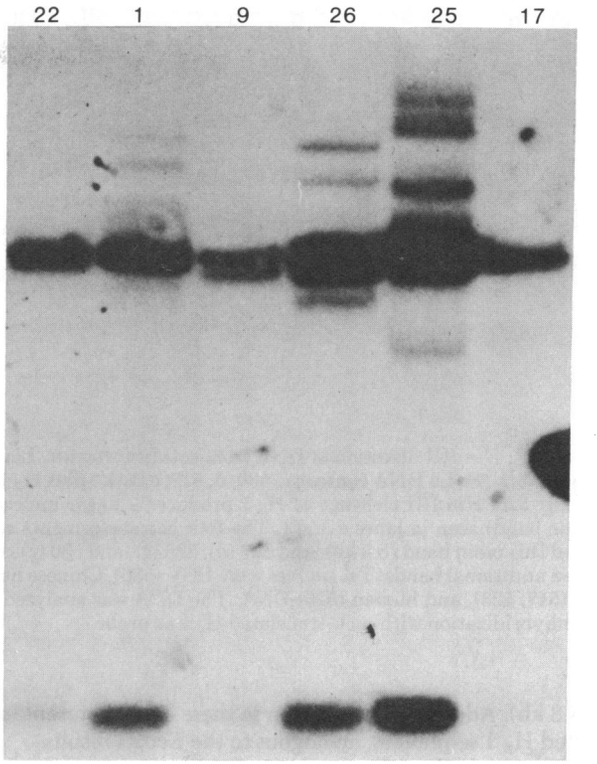

FIG. 2. *Eco*RI digestion of DNA from cotransformants. The lines are 522 (lane 22), 51 (lane 1), 59 (lane 9), 526 (lane 26), 525 (lane 25), and 517 (lane 17). The TK sources were HSV (525), Chinese hamster (51, 59, 517, 522), and human (526) DNA. The two main bands present in almost every line (7.3 kb and 1.5 kb) are identical to the bands seen with *Eco*RI digestion of H_β 1. The DNA was analyzed by filter hybridization using nick-translated H_β 1 as probe.

RESULTS

Cotransformation of Ltk$^-$ cells was accomplished by using three sources of TK: HSV-1, human, and Chinese hamster DNA. The frequencies of recovery of HAT-resistant colonies in the cotransformation experiments using cellular DNA (Table 1) were in the range of those seen by other investigators (7). Most of the H_β 1 molecules used in cotransformation experiments were in the open circular form. We calculate the input ratio of H_β 1 molecules to cellular TK genes to be on the order of 20,000:1. Sixteen of the 52 transformants were expanded and analyzed. DNA was isolated as soon as each line grew to 10^8 cells.

Examination of the restriction map of H_β 1 (Fig. 1) shows that *Eco*RI, *Hin*dIII, and *Kpn* I cleave the molecule twice, once, and not at all, respectively. Initial screening of the 16 TK$^+$ transformants by *Eco*RI digestion and filter hybridization demonstrated that 13 (80%) of the lines contained H_β 1 sequences and that nearly half of these lines had five or more copies per cell. There was no significant correlation between the source of TK and either the frequency of cotransformation or the number of copies of H_β 1 sequences present. One line, 530, had approximately 30 copies of H_β 1 sequences per cell. The most striking finding was that *Eco*RI digestion gave the same two bands in almost every positive line (Fig. 2). These two bands were identical in molecular weight to the bands produced by *Eco*RI digestion of the H_β 1 molecule (7.3 kb and 1.5 kb). Many of the digestions showed additional larger bands representing H_β 1 sequences attached to high molecular weight DNA.

*Hin*dIII analysis of transformants that demonstrated the two main *Eco*RI bands gave a consistent pattern (Fig. 3). Most lines had a band present at a position identical to that of linear H_β

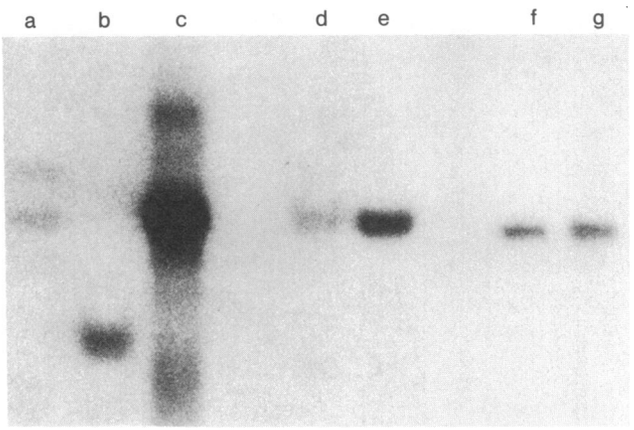

FIG. 3. *Hin*dIII digestion of DNA from cotransformants. Lanes: a, line 523; b, HeLa DNA control; c, 530; d, 517; e, Ltk$^-$ plus H_β 1; f, H_β 1; g, 526. *Hin*dIII cleavage of H_β 1 produces a linear molecule (single band) seen in lanes e and f. The four cotransformants contained this main band (8.8 kb), and 523 (a), 526 (g), and 530 (c) contained additional bands. TK sources were HSV (530), Chinese hamster (517, 523), and human (526) DNA. The DNA was analyzed by filter hybridization with nick-translated H_β 1 as probe.

1 (8.8 kb). Additional bands seen in these lines represent integrated H_β 1 sequences, analogous to the *Eco*RI results.

Kpn I and *Xba* I do not cleave H_β 1, and digestion of the plasmid with either of these two enzymes gave bands corresponding to covalently closed monomers, open circular monomers, and some multimers. Digestion of transformant DNA with *Kpn* I gave a variable pattern (Fig. 4 *A*, *B*, and *C*). At least three lines (523, 525, and 526) out of five analyzed in detail contained a band whose electrophoretic mobility was indistinguishable from that of open circular monomeric plasmid in a background of Ltk$^-$ DNA. These lines contained other bands as well. In addition, line 523 DNA digested with *Xba* I gave a band migrating to the position of open monomeric plasmid circles (Fig. 4*D*).

Four different transformants were maintained in nonselective medium (alpha minimal essential medium without HAT) for 30 days and then reanalyzed as mass populations and as individual subclones. Preliminary results for one of these, line 525, indicated that the restriction patterns with *Eco*RI and *Hin*dIII did not change significantly.

DISCUSSION

We have analyzed H_β 1 sequences transferred by DNA-mediated gene transfer into mouse tissue culture cells. The presence of multiple copies of H_β 1 in the transformants corroborates the findings of Wigler *et al.* (8). H_β 1 DNA represented 3–10% of the DNA used in the transformation and was found in 80% of the TK$^+$ lines analyzed at up to 30 copies per cell. These data indicate that cells which take up and express TK incorporate additional DNA, and that cotransformation is an efficient method for the introduction of nonselected genes into mammalian cells. Nevertheless, cotransformation of genes present at a frequency of one copy per TK gene may be a rare event. We have screened 25 cell lines in which Chinese hamster DNA was used to transform Ltk$^-$ cells, and in no case were any of 15 syntenic or asyntenic donor (Chinese hamster) marker enzymes expressed along with TK. These data are in contrast to those found for chromosome-mediated gene transfer (18, 19).

Evidence for the propagation of unintegrated DNA molecules has been reported (20). In rat hepatoma cells, mouse mammary tumor virus was maintained simultaneously in unintegrated (circular and linear) and in integrated forms. In cell lines carrying many copies of H_β 1 and in cell lines carrying few copies, we have seen a pattern of bands, produced by several restriction enzymes, which is indistinguishable from the pattern produced by digestion of authentic circular plasmid DNA.

*Hin*dIII digestion of integrated monomeric plasmid (Fig. 5) would yield two fragments of varying size, each consisting of a portion of the H_β 1 molecule attached to flanking host sequences. *Hin*dIII digestion of cellular DNA from many transformants produced a band migrating at the position of linearized plasmid (Fig. 3). This can be explained only by site-specific integration at the H_β 1 *Hin*dIII site, the presence of tandem H_β 1 repeats, or the presence of free circular molecules (Fig. 5).

The presence of free circles is favored by the finding that digestion of the DNA of several lines with *Kpn* I, which does not cleave the H_β 1 plasmid, gave a band migrating at the position of open circular plasmid (Fig. 4). Open circular plasmids migrate with an apparent molecular weight of 22 kb. We cannot rule out the possibility that *Kpn* I-digested monomeric or dimeric plasmid molecules attached to cellular DNA coincidentally migrate at this molecular weight, but the presence of bands at 22 kb in several independent lines makes this interpretation unlikely. However, cell lines that gave a *Hin*dIII band characteristic of linear H_β 1 and gave no *Kpn* I band at 22 kb, probably contain some form of tandem H_β 1 repeat.

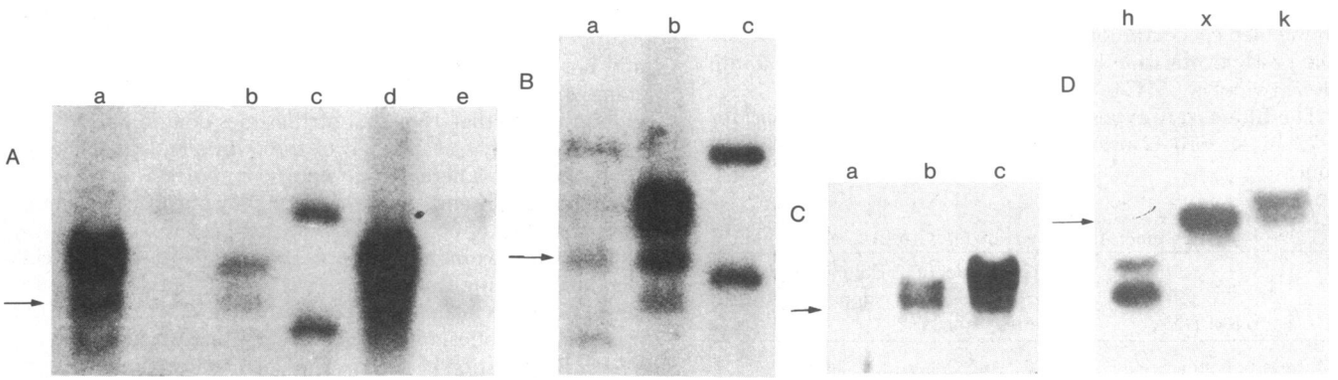

FIG. 4. *Kpn* I and *Xba* I digestion of DNA from cotransformants. All of the digestions for *A*, *B*, and *C* were done with *Kpn* I. (*A*) Lanes: a, line 526; b, 523; c, H_β 1; d, 530; e Ltk$^-$ plus H_β 1. (*B*) Lanes: a, Ltk$^-$ plus H_β 1; b, 526; c, H_β 1. (*C*) Lanes: a, H_β 1; b, 525; c, 526. (*D*) Lanes: h, *Hin*dIII 523; x, *Xba* I 523; k, *Kpn* I 523. The arrow in each photograph indicates the band position corresponding to open monomeric H_β 1 circles electrophoresed in a background of digested Ltk$^-$ DNA (approximately 22 kb). Notice the altered mobility of open circular H_β 1 molecules when electrophoresed with Ltk$^-$ DNA (*B*, lane c vs. lane a). The TK sources are listed in Figs. 2 and 3. The DNA was analyzed by filter hybridization with nick-translated H_β 1 as probe.

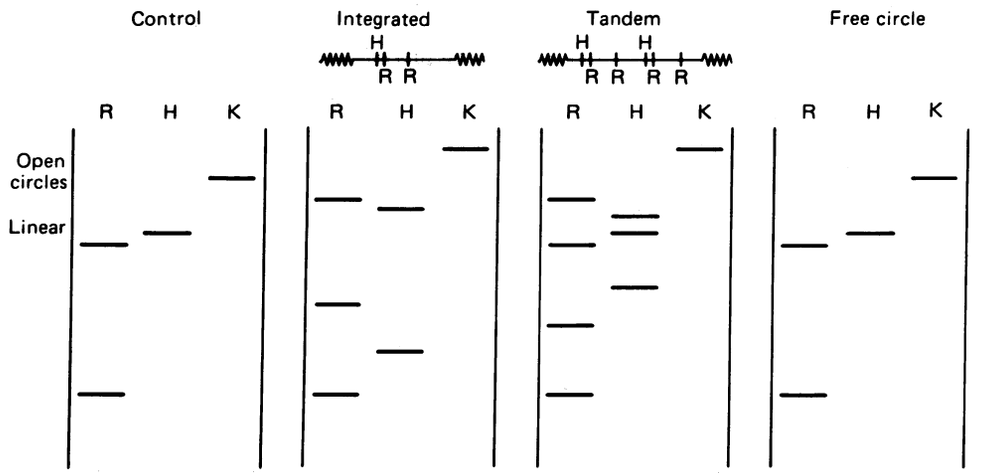

FIG. 5. A diagrammatic representation of band patterns expected after endonuclease digestion of H_β 1 sequences present in transformed cells. The labels "open circles" and "linear" refer to the position of bands representing open circular or linear H_β 1. Control lanes represent circular plasmid which yields: two fragments after *Eco*RI (R) digestion, a full-length linear band after *Hin*dIII (H) digestion, and open circular molecules after *Kpn* I (K) digestion (covalently closed molecules are visualized poorly due to nicking by *Kpn* I and difficulties in blotting). Integrated tracks represent the expected pattern from a single copy of H_β 1 integrated into cellular DNA. *Eco*RI gives one of the two control bands (here the smaller) and two additional bands representing H_β 1 sequences attached to cellular DNA (tail fragments). *Hin*dIII gives two bands, and *Kpn* I gives one band. Integrated tandem repeats (dimer shown here) digested with *Eco*RI would yield several copies of the two control bands plus two tail fragments. *Hin*dIII digestion would give an 8.8-kb fragment (identical to linear H_β 1) and two tail fragments. *Kpn* I would give one high molecular weight band. Free circles would give the same pattern as the control H_β 1.

Analysis of DNA-mediated gene transfer (8, 11) has produced no evidence for site-specific integration.

Many of the cell lines have multiple copies of the plasmid sequences, which confuse the interpretation of band patterns. One cell line with a relatively simple restriction pattern is 523. *Eco*RI digestion (not shown) gave two intense bands corresponding to those from plasmid and two additional faint bands. The intensity of the main bands indicated less than five copies per cell. *Hin*dIII digestion gave three bands, one migrating to the position of linear H_β 1, one larger by several kilobases, and a third very faint band slightly smaller than linear H_β 1 (Fig. 3, lane a). *Kpn* I digestion gave two bands, one migrating to the same location as open circular H_β 1 and one larger (Fig. 4*A*, lane b). Finally, *Xba* I digestion gave one intense band at the position of a free circle (Fig. 4*D*, lane x). Our interpretation of these data is that line 523 contains both free circular and integrated copies of H_β 1. The free circles produced bands indistinguishable from authentic plasmid bands after *Hin*dIII, *Kpn* I, and *Xba* I digestion, whereas the integrated sequences were represented by the other two *Hin*dIII bands and by the larger *Kpn* I band. The *Xba* I band resulting from digestion of the integrated sequence may have been too large to detect. Alternatively, it may have a molecular weight of approximately 22 kb and therefore would comigrate with open circular molecules.

In summary, we believe that we have identified a plasmid, H_β 1, that is capable of existence as an independent circular molecule in mammalian cells. Based solely on restriction analysis with four enzymes, however, we cannot rule out the possibility that all of the bands we saw were derived from integrated (or free) tandem repeats of H_β 1.

If the presence of free H_β 1 circles in our lines is assumed, a straightforward mathematical analysis provides evidence for their replication. The DNA used for the transfer of cellular TK into Ltk^- contained 5×10^6 copies of the TK gene per 10^6 recipient cells. The H_β 1 molecules were present in 20,000-fold excess or 10^5 copies per recipient cell. Even if a cell which took up and expressed TK received 10^7 plasmid molecules (100 times the average per cell), free H_β 1 sequences would be diluted to less than one copy per 10 cells by the time this cell had been expanded to 10^8 cells for DNA isolation. In our hybridization experiments we would not detect sequences present at this concentration. Therefore, the presence of H_β 1 circles at the time of our analysis indicates that amplification has occurred. At this time we cannot distinguish between the replication of autonomous circular molecules and the generation of unintegrated molecules from integrated sequences, possibly concatamers.

If H_β 1 can propagate itself in mammalian cells without chromosomal integration, it will provide two promising new directions of research. Restriction fragments of H_β 1 can be cloned and assayed in cotransformation experiments to localize and eventually to sequence a functional mammalian origin of replication. In addition, the isolation of a plasmid capable of independent replication in both bacterial and mammalian cells would represent a new advance in the ability to transfer and propagate defined sequences in mammalian cells.

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