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Deoxyribonucleic Acid-Mediated Gene Transfer in Mammalian Cells: Molecular Analysis of Unstable Transformants and Their Progression to Stability

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To elucidate mechanisms involved in deoxyribonucleic acid-mediated gene transfer, we transferred the herpes simplex virus thymidine kinase gene (TK) into mouse Ltk$^-$ cells. Independent TK$^+$ clones (transformants) and derivatives of each were tested for phenotypic expression and the presence and arrangement of TK sequences. Initially, transformants expressed viral TK unstably, with 10% of the cells in each generation losing both the TK$^+$ phenotype and virally derived TK sequences. After a prolonged period in culture, stable subpopulations arose from which the TK$^+$ phenotype and viral sequences were no longer lost at detectable frequency. Analysis of unstable cell populations indicated that individual viral deoxyribonucleic acid molecules were reduced in size, but were linked to other deoxyribonucleic acid to form molecules large enough to be precipitated in a Hirt fractionation. We term these molecules transgenomes. Analysis of independent unstable subclones derived from the primary transformants demonstrated that individual transgenomes could contain multiple copies of the viral TK sequences. Recipient cell lines frequently possessed more than one type of transgenome and possibly multiple copies per cell of each type. Stable derivatives possessed only one of the transgenomes present in the unstable parent, and these sequences were associated with a recipient cell chromosome.

The ability of mammalian cells to take up exogenous deoxyribonucleic acid (DNA) and to express genes encoded by that DNA has been well established. Graham and van der Eb (8) showed that viral DNA complexed with a calcium phosphate precipitate could be used to transfect cells. Wigler et al. (21) extended these findings by demonstrating genetic transformation of cells using a thymidine kinase gene (TK) purified from herpes simplex virus (HSV) DNA. They demonstrated a requirement for calcium phosphate precipitation of the DNA and showed that excess carrier DNA greatly enhanced transformation efficiency. More recently, it has been shown that DNA carrying a genetic marker, added together with DNA possessing a selectable genetic function, could be cotransferred at high efficiency into the transformed cell (10, 22). The mechanisms which mediate the DNA transformation of mammalian cells, however, remain poorly understood, as is the biochemical fate of the transferred material. It has been suggested that the selected gene, other cotransferred nonselected genes, and carrier DNA may actually join together physically in the transformed cell. In our previous reports we have termed such entities "transgenomes" (12), and Wigler et al. have referred to what is most probably the same entity as a "pekalesome" (20). In this report, we present molecular and cytological evidence which supports the existence of such entities and which provides new information on their mode of origin and their probable structure.

In our experimental system, we used a 3.5-kilobase (kb) *Bam*HI-derived HSV genomic fragment containing the TK gene. For convenience, the fragment has been cloned in pBR322 (3), and the entire plasmid, termed pTKx-l, was used as the selectable marker. We used mouse Ltk$^-$ cells as recipients and DNA extracted from these cells as carrier. In this report, we confine our remarks to transformants produced by linear pTKx-l fragments mixed with excess Ltk$^-$ carrier DNA.

In previous studies it has been shown that transformed cells under nonselective conditions may lose the selectable marker at a characteristic rate or may retain it indefinitely (1, 7, 21). We term these two cell types unstable and stable transformants. In the experiments reported here, we have characterized our transformed cell populations extensively with regard to the stability of the TK marker. We have found that

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instability precedes stability, and that stable lines rarely revert to instability. Thus, by classifying the transformed populations as unstable and stable, and by following the transition from instability to stability within a clonally derived population of transformed cells, we are able to define a temporal sequence of events. We believe this progression is extremely important in determining transitions in the structure, copy number, and type of transgenomes. Our earlier reports on mammalian cell transformation systems have advanced the hypothesis that stability can be explained by the integration of an autonomous transgenome (unstable state) into a host cell chromosome (stable state). Here we provide additional evidence to support the association of the transgenome with a recipient cell chromosome in the stable cells.

The results described here add a new feature to this model. They show that within a clonally derived unstable population, a number of molecularly distinct transgenomes may exist, and these may become segregated into separate cell populations during the progression of the transformed populations which carry a single type of transgenome. The dissection of transformed cell populations in this way considerably simplifies the molecular analysis of the overall transformation process and of the molecular and genetic properties of the transgenome and, for the first time, allows precise molecular models to be constructed at different stages of the transformation process.

MATERIALS AND METHODS

Cell culture. Cell lines were maintained as monolayer cultures at 37°C in minimal essential medium, alpha modification (Gibco), supplemented with 5% fetal calf serum (Flow Labs). TK^+ cell lines were maintained in the same medium supplemented with HAT (13). For transfer, 10^6 cells of the TK^- mouse cell line Ltk^- were plated per 75-cm^2 flask 24 h before the addition of DNA. Four hours after the DNA addition, the medium and DNA were removed and replaced with fresh, nonselective medium. Selective HAT medium was added 30 h after DNA addition. The cells were fed 24 h later and subsequently every 3 days.

TK^+ colonies, which appeared in about 2 weeks, were picked, grown to 10^6 cells, and divided into two fractions. One fraction was expanded and frozen for storage. The second fraction was expanded to 10^8 cells and used for DNA isolation. A portion of the cells from this second fraction was maintained in culture in both selective and nonselective media and was used for stability testing and for the isolation of stable derivatives. Stable derivatives were identified by maintaining cells in nonselective medium for 60 days, at which time 10^5 cells were returned to selective medium. TK^+ colonies were tested for stability as described below. One stable derivative of each unstable parent (designated by the suffix A) was expanded, and DNA was isolated.

To characterize stability of TK expression, the line to be tested was removed from HAT selective medium and maintained for 1 week in HT medium and subsequently in unsupplemented alpha medium. At day 0 (the day at which the cells were removed from selection) and periodically thereafter 400 cells were plated in two 25-cm^2 flasks in HAT medium and an equal number were plated in two flasks in HT (nonselective) medium. Ten days after plating, the flasks were stained and colonies were counted.

For selection against TK expression, cells were grown nonselectively for a period of 2 weeks and then were transferred to medium containing 30 μg of 5-bromodeoxyuridine per ml. The next day, cells were exposed to a Sylvania F15T8 ultraviolet lamp for 30 min at a distance of 18 cm. The ultraviolet exposure was repeated 1 day later. Cells which grew into colonies in 5-bromodeoxyuridine-supplemented medium were tested for HAT resistance. TK^- derivatives of each unstable and stable cell line were designated by the suffix BU. One derivative of each line was saved.

Formation of the calcium phosphate precipitate. A 3-ng sample of *Hin*dIII-digested pTKx-1 plasmid and 30 μg of Ltk^- DNA were diluted into 1.5 ml of 250 mM $CaCl_2$ (Mallinkrodt, anhydrous)–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.1. This mixture was added dropwise with agitation to 1.5 ml of a solution of 280 mM NaCl–50 mM HEPES–1.5 mM Na_2HPO_4, pH 7.1. The precipitate was allowed to form for 30 min at room temperature, and 1 ml was added directly to the medium of each of three 75-cm^2 flasks.

Microcell fusion. TK^+ mouse cell lines were plated on plastic disks as described previously (5) and exposed to 0.05 μg of colemid per ml for 40 h. Loosely attached cells were removed by a preliminary centrifugation at 14,500 × *g*, and microcells were isolated by centrifugation at 39,000 × *g* in medium containing 10 μg of cytochalasin B per ml. Routinely, 1×10^6 to 3×10^6 microcells were isolated from 16 disks and were fused with an equal number of Chinese hamster RJK cells by using polyethylene glycol (6). HAT selection was applied 30 h after the fusion.

DNA isolation. High-molecular-weight cellular DNA was isolated as described by Wigler et al. (21). Plasmid pTKx-1 was a gift of W. Summers and was grown in LE 392 under P2-EK1 conditions in accordance with National Institutes of Health guidelines. DNA was isolated as described by Wilson et al. (23). Hirt extraction of cell lines was performed as described by Pellicer et al. (14).

Filter hybridization analysis. Restriction endonucleases *Hind*-III and *Eco*RI were purchased from Boehringer Mannheim. *Xba*I, *Hin*cII, and *Pvu*II were purchased from New England Biolabs. DNA was digested at 1 unit of enzyme per μg of DNA for 10 h. The use of 6 U/μg for 10 h did not result in a change in the band patterns. A 15-μg sample of cellular or Hirt precipitate DNA was loaded in each track. To maximize the chances of finding sequences in the Hirt supernatant, a sample isolated from 10^7 cells was loaded in each track. Agarose gel electrophoresis and transfer of DNA to nitrocellulose filters were per-

formed as described previously (10). Filter hybridizations were performed in the presence of dextran sulfate as described by Wahl et al. (19), with the modifications described previously (10).

RESULTS

Plasmid pTKx-1 consists of a 3.5-kb fragment of viral DNA containing the TK gene of HSV type 1 inserted into the *Bam*HI site of pBR322 (3) (Fig. 1). This plasmid, cut with *Hin*dIII to generate a linear molecule, was mixed with high-molecular-weight carrier DNA and coprecipitated with calcium phosphate onto cells of the TK^- mouse cell line Ltk^-. Three independent TK^+ colonies and derivatives of each were examined in detail. In each case, we demonstrated the viral nature of the thymidine kinase enzyme (17).

When grown in nonselective medium, each of the original TK^+ unstable transformants, termed LHI 1, LHI 2, and LHI 3, became TK^- at a rate of approximately 10% per day (Fig. 2). After 60 days in nonselective medium, clonally derived subpopulations of each line which still retained the TK^+ phenotype were isolated and retested for stability of expression. Each subpopulation, designated by the suffix A, was found to express TK stably (Fig. 2).

DNA isolated from recipient lines LHI 1, LHI 2, and LHI 3 and from derivatives of each which could no longer grow in selective medium was digested with *Hin*dIII, *Xba*I, and *Eco*RI and analyzed by Southern blot filter hybridization with the pTKx-1 plasmid as probe. No sequences homologous to the probe were seen in the TK^- derivatives, indicating that loss of expression of the selected gene from the unstable population was a result of the loss of the gene sequences.

Reduction in size of plasmid pTKx-1. Filter hybridization analysis of *Hin*dIII or *Xba*I-digested DNA from each of the unstable lines LHI 1, LHI 2, and LHI 3 demonstrated the presence of bands smaller than 7.9 kb. Since neither *Hin*dIII nor *Xba*I recognizes any sequences within the linear 7.9-kb plasmid pTKx-1, these results indicate that some of the plasmid sequences had been lost from each of these cell lines (Fig. 3, 4, and 6).

The size of the single *Xba*I band present in LHI 1A was 5.9 kb (Fig. 3). Since the sites which generated this band must have lain in DNA sequences flanking the plasmid (*Xba*I recognizes no internal sites), there must have been less than 5.9 kb of the 7.9-kb plasmid remaining in this cell line. Digestion of *Hin*dIII-linearized pTKx-1 with *Eco*RI generated two internal fragments of 2.3 and 4.6 kb. Digestion of LHI 1A DNA with *Eco*RI generated two bands of 3.2 and 9.4 kb (Fig. 3). The absence of both internal fragments indicates that at least two of the three

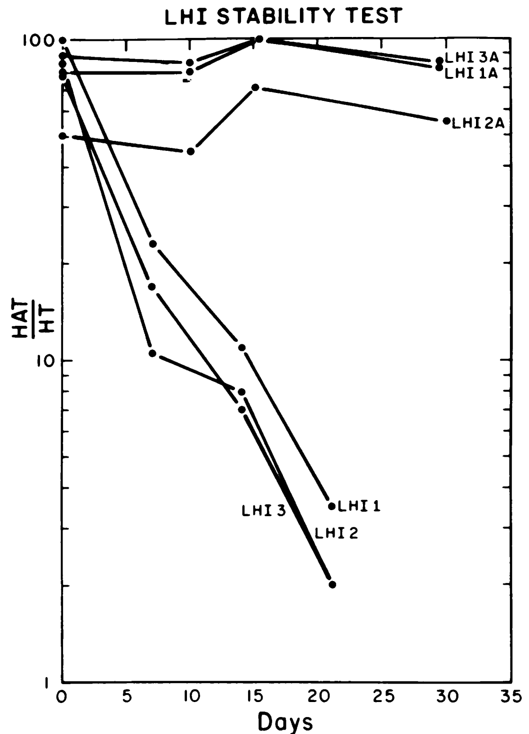

FIG. 2. *Analysis of the stability of transformant cell lines. Each cell line was grown in nonselective medium. Periodically, 400 cells were plated in selective (HAT) and nonselective (HT) medium, and the number of colonies which arose in each medium was counted. The primary transformants are designated LHI 1, LHI 2, and LHI 3. Stable subpopulations of each are designated by the suffix A.*

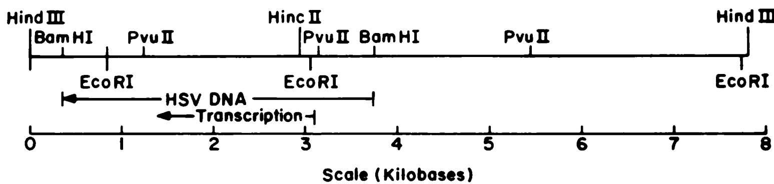

FIG. 1. *Partial restriction map of plasmid pTKx-1. A 3.5-kb fragment of HSV type 1 DNA containing the viral TK gene has been inserted into the BamHI site of plasmid pBR322 (3).*

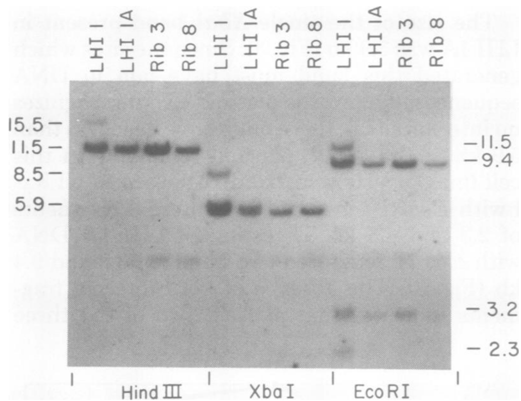

FIG. 3. *Filter hybridization analysis of DNA from LHI 1 and derivatives. LHI 1 is the parental unstable transformant, LHI 1A is a stable derivative, and Rib 3 and Rib 8 are two microcell hybrids formed between LHI 1A and the TK⁻ Chinese hamster line RJK.*

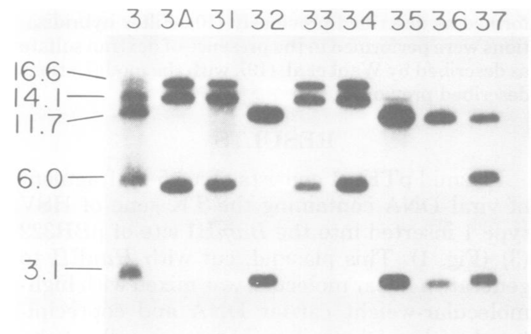

FIG. 4. *Filter hybridization analysis of DNA from LHI 3 and derivatives. LHI 3 is an unstable transformant, LHI 3A is a stable derivative, and lines LHI 31 through LHI 37 are unstable subclones of LHI 3. All DNA samples were digested with HindIII.*

*Eco*RI sites in the plasmid were lost from this cell line. Digestion of LHI 1A DNA with *Pvu*II yielded two bands of 3.9 and 2.9 kb. The expected internal fragments of 2.0 and 2.3 kb (Fig. 1) again were not seen (data not shown), indicating that at least two of the three *Pvu*II sites were lost. These data indicate that at least 3.6 kb of plasmid sequences spanning the region between the two external *Pvu*II sites and the ends of the molecule (as drawn in Fig. 1) were lost during the transformation process.

*Xba*I digestion of LHI 1 DNA generated a band of 8.5 kb as well as the 5.9-kb band retained in the stable derivative LHI 1A. *Eco*RI digestion of LHI 1 DNA generated four bands, two of which are indistinguishable from those in LHI 1A. One of the two additional bands was 2.3 kb, the size of one of the expected internal fragments. These data suggest that the plasmid sequences represented by one of the two *Xba*I-derived bands contained at least two *Eco*RI sites (to yield the 2.3-kb internal fragment), whereas the sequences represented by the other band did not possess both *Eco*RI sites. Therefore, the two bands present after *Hin*dIII or *Xba*I digestion of LHI 1 DNA contained different subsets of the original plasmid sequences.

Of the five bands seen after *Hin*dIII digestion of LHI 3 DNA (Fig. 4), at least two lost the two *Eco*RI sites closest to the ends of the molecule as drawn in Fig. 1. This was demonstrated by digestion of DNA from LHI 32, a subclone of LHI 3 which retained two of the five *Hin*dIII-derived bands present in the parental line. *Eco*RI digestion of LHI 32 failed to yield the 2.3- or 4.6-kb internal fragments, indicating that both of the plasmid sequences present in this line lost the external *Eco*RI sites. DNA from LHI 34, an independent subclone of LHI 3, retained three of the parental *Hin*dIII bands. *Eco*RI digestion of LHI 34 DNA yielded a 2.3-kb band, indicating that the sequences represented by one of the *Hin*dIII bands in this line possessed at least one of the external *Eco*RI sites. Since the bands present in LHI 32 and LHI 34 all are present in the clonally derived line LHI 3, these data suggest that at least two different subsets of the plasmid sequences were present in LHI 3. To demonstrate that the 2.3-kb band present in LHI 34 was indeed an internal fragment, we used purified viral TK fragment and pBR322 as probes of this DNA. The 2.3-kb fragment hybridized to the viral TK fragment, but not to pBR322, indicating that it was derived from TK sequences and was most probably the result of cleavage at the two *Eco*RI sites within the viral sequences.

Ligation of high-molecular-weight DNA to plasmid pTKx-l in unstable cell lines. The sizes of many of the bands seen after *Hin*dIII or *Xba*I digestion of DNA from the unstable transformants LHI 1, LHI 2, and LHI 3 were greater than 7.9 kb. The two bands present in *Hin*dIII-digested LHI I DNA were 15.5 and 11.5 kb, those in LHI 2 ranged from 19 to 7.6 kb, and those in LHI 3 were 16.6, 14.1, 11.7, 6.0, and 3.1 kb. Since plasmid pTKx-l is 7.9 kb, these data indicate the presence either of concatemeric plasmid sequences or of an association of the plasmid with other DNA. Digestion of LHI 1 DNA with *Xba*I generated two bands of 8.5 and 5.9 kb, smaller than the bands generated by *Hin*dIII digestion of the same DNA. Since *Xba*I does not cleave within the plasmid, the larger *Hin*dIII-derived bands must be a result of the association of the plasmid with high-molecular-weight DNA.

We performed Hirt extractions on cells of LHI 1, LHI 2, and LHI 3 to determine the size of the DNA linked to the plasmid. DNA from precipitate and supernatant fractions was digested with *Hin*dIII, *Xba*I, and *Eco*RI and analyzed for the presence of plasmid sequences. In each case the pattern of bands seen in DNA from the Hirt precipitate was similar to that seen in total nuclear DNA (LHI 1 analysis shown in Fig. 5; LHI 2 and 3 not shown). The differences in band intensity are a reflection of differences in the amount of DNA loaded in the whole-cell tracks versus the Hirt precipitate tracks. Even after long overexposures, no sequences complementary to plasmid probe were seen in the Hirt supernatant fraction from any of the lines, indicating that the TK sequences in these unstable cell lines were associated with high-molecular-weight DNA.

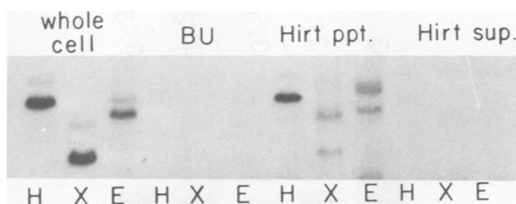

FIG. 5. *Hirt analysis of LHI 1. Sequences homologous to the pTKx-1 probe are seen in whole-cell DNA and in DNA from the Hirt precipitate. No sequences are seen in the Hirt supernatant or in whole-cell DNA isolated from a subpopulation of LHI 1 which survived growth in medium containing 5-bromodeoxyuridine. DNA samples were digested with HindIII (H), XbaI (X), and EcoRI (R).*

Existence of independent and different transgenomes in a single transformed clone. Digestion of LHI 2 DNA with *Hin*dIII yielded eight bands complementary to the plasmid probe (Fig. 6A), indicating that the plasmid sequences were located at a minimum of eight sites in the genome of the clonally derived recipient cell population. DNA isolated from a stable derivative, LHI 2A, possessed six of the eight bands (Fig. 6A). These data suggested that the six bands were linked on the same molecule and were retained as the result of a single stabilization event. To test this hypothesis we isolated subclones of the unstable line LHI 2. Each of the subclones continued to express TK unstably (not shown). Analysis of the DNA from six subclones is shown in Fig. 6. Subclone LHI 24, derived

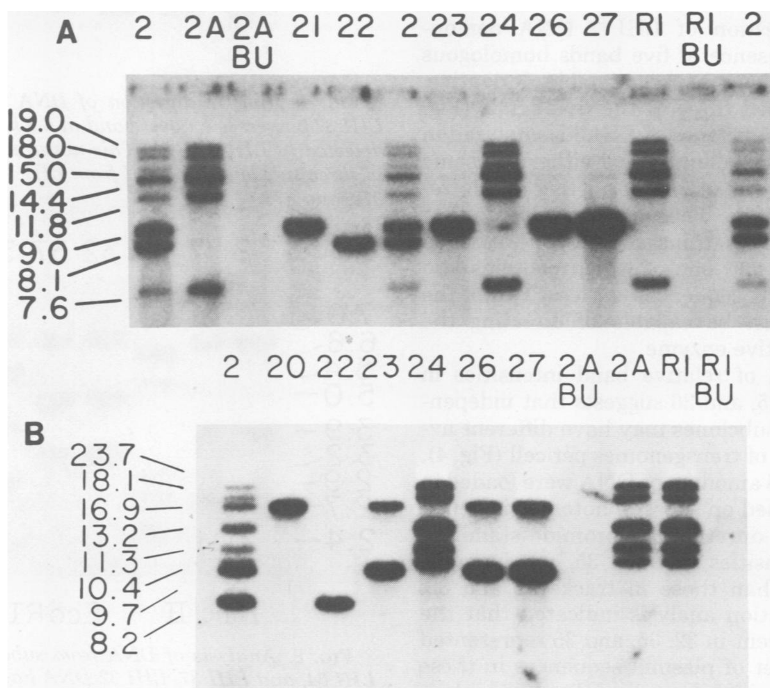

FIG. 6. *Filter hybridization analysis of DNA of LHI 2 and derivatives. LHI 2 is an unstable transformant, LHI 2A is a stable derivative, and lines LHI 21 through 27 are unstable subclones of LHI 2. Line R1 is a microcell hybrid formed between LHI 2A and the TK⁻ Chinese hamster line RJK. Lines designated BU have been selected for the loss of TK by their resistance to 5-bromodeoxyuridine. (A) HindIII digestion; (B) XbaI digestion.*

from LHI 2, had a pattern of bands indistinguishable from that of the stable derivative, LHI 2A. Additionally, none of the other subclones of LHI 2 possessed any of those six bands, suggesting that the sequences represented by these bands were linked on the same molecule and were unlinked to the sequences represented by the remaining two bands. This conclusion was supported by the transfer of all six bands from LHI 2A into line RJK by microcell fusion (Fig. 6) (5). Other subclones possessed one or the other of the two bands not present in LHI 2A.

*Hin*dIII digestion of DNA from subclones 23, 26, and 27 yielded one band whereas *Xba*I digestion of the same DNAs yielded two bands (Fig. 6). Since neither of these enzymes cleaves within the plasmid sequences, the single *Hin*dIII band must represent two copies of the plasmid sequences. Thus the eight *Hin*dIII bands in the parental line LHI 2 indicate that this line possesses TK sequences at a minimum of nine sites. In each subclone, at least one of the bands must represent an active TK gene for the cells to survive in selective medium. Since subclones 22, 23, and 24, for example, possess no bands in common, there must have been at least three active or potentially active copies of the HSV TK gene in the parental cell line LHI 2.

*Hin*dIII digestion of LHI 3 DNA demonstrated the presence of five bands homologous to the TK probe, whereas a stable derivative, LHI 3A, retained three of the five bands (Fig. 4). DNA from subclones of LHI 3 is analyzed in Fig. 4. Each subclone retained either the same three bands as the stable derivative or the remaining two bands. These results indicate that LHI 3 possesses two transgenomes, one of which contains three and one of which contains two copies of the TK sequences. At least two of the five copies must be capable of directing the synthesis of active enzyme.

Examination of relative band intensities in subclones 32, 35, and 36 suggests that independent unstable subclones may have different average numbers of transgenomes per cell (Fig. 4). Although equal amounts of DNA were loaded in each track (based on spectrophotometric determinations and on ethidium bromide staining), the band intensities in track 35, for example, were greater than those in tracks 32 and 36. Further restriction analysis indicated that the two bands present in 32, 35, and 36 represented the same subset of plasmid sequences in these three subclones (not shown), so that band intensity may be correlated with copy number.

Subclone LHI 37 contained a novel band at 6.8 kb which was absent in the original line, LHI 3, and in all other subclones. Additionally, the 11.7-kb band was diminished in intensity with respect to the 3.1-kb band (compare subclones 32, 35, 36, and 37; Fig. 4). The novel 6.8-kb band was distinct from the 6.0-kb band seen in the parental line LHI 3, as demonstrated by its slower mobility when *Hin*dIII-digested DNA from LHI 3 and LHI 37 was run in adjacent tracks (Fig. 7). Suprisingly, we detected no difference in the band pattern of subclones LHI 37 and LHI 32 after digestion of the DNAs with *Hin*cII or *Eco*RI (Fig. 8). These data are con-

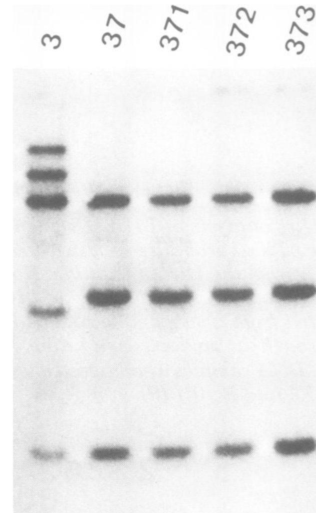

FIG. 7. *HindIII digestion of DNA from LHI 37. LHI 37 possesses a novel band at 6.8 kb which is not detected in LHI 3 DNA. This novel band is present in three subclones isolated from LHI 37, termed 371, 372, and 373.*

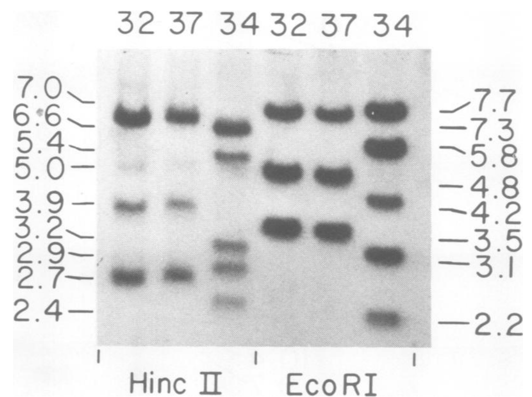

FIG. 8. *Analysis of DNA from subclones LHI 32, LHI 34, and LHI 37. LHI 32 DNA had two HindIII-derived bands, and LHI 34 DNA had three. LHI 37 DNA had the two bands present in LHI 32 plus a novel band. HincII and EcoRI digestion of DNA from these lines yielded no differences between LHI 32 and LHI 37. Additionally, no common bands were seen between LHI 32 or LHI 37 and LHI 34.*

sistent with the novel band in LHI 37 being derived from a modification of the sequences flanking the 11.7-kb band which affected a distal *Hin*dIII site but which did not affect more proximal *Eco*RI or *Hin*cII sites. Analysis of subclones of LHI 37 demonstrated the presence of both the 11.7-kb band and the novel band in DNA isolated from three stable subclones (Fig. 7). When the purified viral TK fragment, rather than the pTKx-l plasmid, was used to probe the DNAs of LHI 3 and its subclones after *Hin*dIII digestion, the 16.6-, 14.1-, and 11.7-,kb bands appeared, whereas the 6.0- and 3.1-kb bands showed little or no homology. The novel 6.8-kb band in LHI 37 did appear with the TK fragment as probe (not shown). Since the 16.6- and 14.1-kb bands are absent in LHI 37, the 6.8-kb band must have arisen from a modification of the sequences flanking the 11.7-kb band.

Integration of the transgenome into a specific host cell chromosome. LHI 1A was used as a donor in microcell fusion experiments with the TK$^-$ Chinese hamster line RJK as a recipient. A summary of the analysis of four independently derived hybrids is shown in Fig. 9. Cells of three of the TK$^+$ hybrids contained one or two mouse chromosomes per cell and had only one mouse chromosome in common. The fourth hybrid contained no identifiable mouse chromosome and was probably the result of fragmentation of donor material. Analysis of DNA from two of the hybrids is shown in Fig. 3. In each case, the band pattern seen in DNA from hybrid cells was indistinguishable from that of the donor LHI 1A. After selection of the hybrids against TK, the common chromosome was lost from the hybrids and the TK sequences were no longer detectable in cellular DNA (not shown). These data indicate that the TK sequences present in LHI 1A are associated with a particular mouse chromosome.

DISCUSSION

We have characterized three cell lines transformed with the HSV TK gene cloned in pBR322. Based on our analyses of these lines,

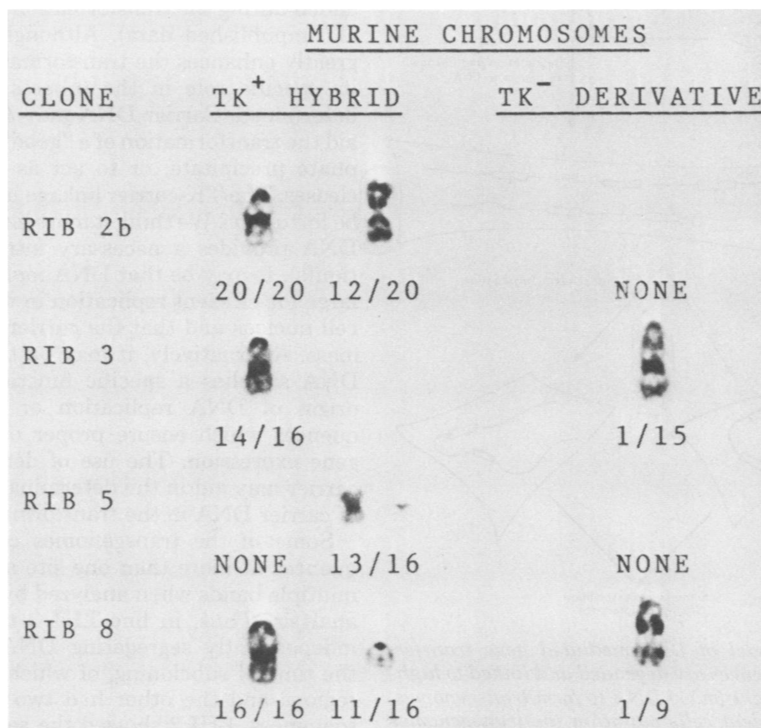

FIG. 9. *Analysis of murine chromosomes in microcell hybrids between LHI 1A and RJK. Microcells isolated from LHI 1A were fused with TK$^-$ deficient Chinese hamster cell line RJK. Karyotypic and isozyme analysis of four independent hybrids was performed. One murine chromosome was present at a high frequency in Rib 2b, Rib 3, and Rib 8 and was absent from TK$^-$ derivatives of each. Rib 5 contained chromosomal fragments whose origin could not be determined but which disappeared on back-selection. These data support the association of the TK sequences with the mouse chromosome common to Rib 2b, Rib 3, and Rib 8. No detectable murine isozymes were encoded by this chromosome. Numbers indicate the number of cells with the chromosome and the total number of cells karyotyped.*

we propose a model for the gene transfer process as outlined in Fig. 10. Recipient cell lines initially express the transferred phenotype unstably. In this unstable state the donor plasmid sequences have been reduced in size, and the remaining sequences are linked to high-molecular-weight DNA. Each high-molecular-weight molecule (called a transgenome) may possess TK sequences at multiple sites. Furthermore, each clonally derived population may have two or more different types of transgenomes and multiple copies of each. The transgenomes are maintained unstably, and eventually all are lost when the population is maintained under nonselective conditions. Maintenance of the cells under selective conditions eliminates those cells which have lost all their transgenomes. Analysis of subclones demonstrates that most cells possess only one of the transgenome types present in the parental cell line.

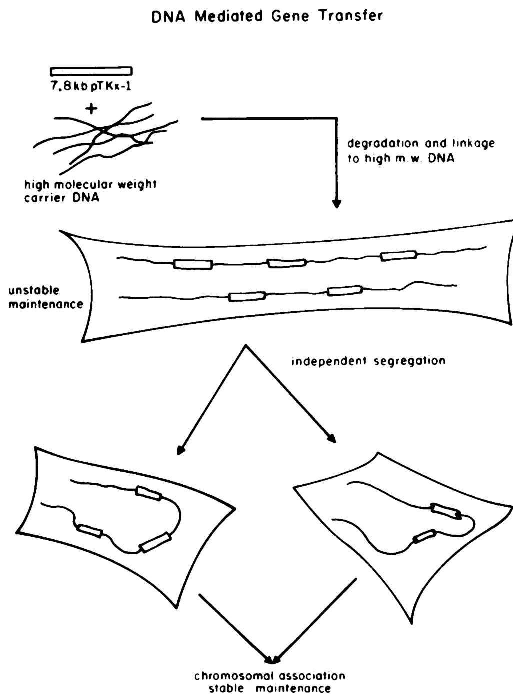

FIG. 10. *Model of DNA-mediated gene transfer. Input TK sequences are degraded and linked to high-molecular-weight (m.w.) DNA to form transgenomes. Initially, recipient cells maintain the transgenomes unstably. Each transgenome may have more than one copy of the TK sequences, and each clonally derived cell line may have more than one type of transgenome. Additionally, multiple copies per cell of each type of transgenome may exist. The transgenomes segregate in daughter cells, eventually become associated with a recipient cell chromosome, and subsequently are maintained stably.*

In the three unstable lines described here, and in a large number of other lines isolated after chromosome and DNA-mediated gene transfer, the transferred phenotype is lost from the population with first-order kinetics (11, 12, 16; Huttner, Scangos, and Ruddle, unpublished data). We have found that populations which deviate from a first-order loss rate are mixed: some of the cells express the selected marker stably, and some continue to lose the marker. In a mixed population, the stable cells eventually predominate in selective medium. (For a complete discussion of stability, see reference 11.)

Although previous studies have demonstrated the instability of recipient cell lines after DNA-mediated gene transfer)1, 7, 21), in no case was the physical state of the transferred gene correlated with a thoroughly characterized phenotype. We have found that the 7.9-kb plasmid is associated with high-molecular-weight DNA even in the unstable state.

Evidence exists which indicates that the DNA linked to the TK gene on the transgenome is derived predominantly from the carrier DNA added during the transformation (15; Huttner et al., unpublished data). Although carrier DNA greatly enhances the transformation frequency, its specific role in the process has not been determined. Carrier DNA may act passively to aid the transformation of a "good" calcium phosphate precipitate, or to act as a sink for nucleases. The TK-carrier linkage in that case may be fortuitous. We think it more likely that carrier DNA provides a necessary intracellular function(s). It may be that DNA molecules must be large for efficient replication in the mammalian cell nucleus and that the carrier DNA supplies mass. Alternatively, it may be that the carrier DNA supplies a specific function such as an origin of DNA replication or nucleotide sequences which ensure proper organization for gene expression. The use of defined DNAs as carrier may aid in the determination of the role of carrier DNA in the transformation process.

Some of the transgenomes contain TK sequences at more than one site and give rise to multiple bands when analyzed by Southern blot analysis. Thus, in line LHI 3 there were two independently segregating DNA molecules at the time of subcloning, of which one had three regions and the other had two regions of TK sequences. LHI 2 showed the same pattern except that there appeared to be three independent transgenomes. In both instances, our observations define the state of the transgenomes in the cells at one point after transformation. We cannot determine whether one copy of the TK plasmid present in an ancestral cell was rearranged and amplified, or whether the mul-

tiple copies of plasmid sequences are the result of independent plasmid molecules associating in different ways with high-molecular-weight DNA. Additionally, we do not know whether the multiple transgenomes that we see represent the original state of the transgenomes in the recipient cells, or may represent fragments derived from a larger, precursor transgenome. This latter interpretation seems unlikely, because on extended cultivation, the transgenomes that we see appear to be maintained without further fragmentation.

We have found only one type of transgenome retained in each unstable subclone. We believe this is so because one transgenome containing an active TK gene is sufficient to allow growth in selective medium, and each is retained unstably. Therefore, a cell with multiple transgenomes will rapidly generate daughters which have only a subset of the transgenomes present in the parent, and this process will continue until each cell has only one type of transgenome.

Analysis of chromosome-mediated gene transfer indicates that multiple copies of a single type of transgenome do exist in unstable cell lines (16). In such cell lines, the instability appears to be due to the lack of centromeric function of the transferred chromosomal fragment and subsequent lack of distribution at mitosis (11). If the transferred fragments are segregating randomly at mitosis, a cell line must possess between three and four such fragments to show a 10% per generation loss rate. This number is consistent with enzymatic determinations and analyses of DNA isolated from such lines (16). If we assume that the transgenomes formed after DNA-mediated gene transfer also lack centromeric function, unstable cell lines must also possess between three and four independently segregating transgenomes per cell to explain their 10% loss rate.

The term competency has been used to describe a physiological state of recipient cells in which they are more likely to take up and express exogenous DNA (18, 21). We feel that this phenomenon by itself does not account for the low frequency of transformation we see. Calcium phosphate precipitation under conditions designed to stimulate gene transfer results in the uptake of DNA by most cells (A. Loyter, G. Scangos, D. Juricek, D. Keene, and F. Ruddle, unpublished data). Each transformed cell population we have characterized contains a large transgenome which consists of multiple independent donor fragments. It may be that only those cells which have received the proper mix of donor fragments and have subsequently joined them in the proper arrangement for maintenance, replication, protection from nuclear exonucleases and endonucleases, and distribution at mitosis will survive in selective medium. We feel that these factors play a significant role in determining the transformation frequency.

We have identified a chromosome with which the TK sequences are associated in LHI 1A by microcell fusion with the TK^- Chinese hamster line RJK. These data indicate that stabilization can occur as the result of the association of the TK gene with a recipient cell chromosome. Thus, the data are consistent with results previously obtained in studies of chromosome-mediated gene transfer (4, 12) and for ultraviolet-inactivated HSV (17). We have been repeatedly unable to demonstrate a chromosomal association by microcell fusion for the TK sequences in the unstable cell lines LHI 1 and LHI 2. We have obtained preliminary evidence that in the unstable state the transgenomes exist independently of the recipient chromosomes (Klobutcher, Scangos, and Ruddle, unpublished data). Since the transgenomes are composed at least in part of carrier DNA isolated from recipient cells, we cannot determine whether chromosomal association is a result of site-specific integration of homologous sequences or is a more nearly random event. Again, the use of defined carrier DNA or of no carrier in transfer experiments may provide an answer to this question.

We view transformation as a sequential process in which the state of donor material immediately after transfer may differ substantially from its state after a period in culture. Recognition of these differences is important for an understanding of the process and for the process to be useful as a tool in the genetic analysis of mammalian cells.

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