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MOLECULAR AND CELLULAR BIOLOGY, Nov. 1982, p. 1372–1387
0270-7306/82/111372-16$02.00/0
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Vol. 2, No. 11

Patterns of Integration of DNA Microinjected into Cultured Mammalian Cells: Evidence for Homologous Recombination Between Injected Plasmid DNA Molecules

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Received 1 June 1982/Accepted 23 July 1982

We examined the fate of DNA microinjected into nuclei of cultured mammalian cells. The sequence composition and the physical form of the vector carrying the selectable gene affected the efficiency of DNA-mediated transformation. Introduction of sequences near the simian virus 40 origin of DNA replication or in the long terminal repeat of avian sarcoma provirus into a recombinant plasmid containing the herpes simplex virus thymidine kinase gene (pBR322/HSV-tk) enhanced the frequency of transformation of LMtk$^-$ and RAT-2tk$^-$ cells to the TK$^+$ phenotype 20- to 40-fold. In cells receiving injections of only a few plasmid DNA molecules, the transformation frequency was 40-fold higher after injection of linear molecules than after injection of supercoiled molecules. By controlling the number of gene copies injected into a recipient cell, we could obtain transformants containing a single copy or as many as 50 to 100 copies of the selectable gene. Multiple copies of the transforming gene were not scattered throughout the host genome but were integrated as a concatemer at one or a very few sites in the host chromosome. Independent transformants contained the donated genes in different chromosomes. The orientation of the gene copies within the concatemer was not random; rather, the copies were organized as tandem head-to-tail arrays. By analyzing transformants obtained by coinjecting two vectors which were identical except that in one a portion of the vector was inverted, we were able to conclude that the head-to-tail concatemers were generated predominantly by homologous recombination. Surprisingly, these head-to-tail concatemers were found in transformants obtained by injecting either supercoiled or linear plasmid DNA. Even though we demonstrated that cultured mammalian cells contain the enzymes for ligating two DNA molecules very efficiently irrespective of the sequences or topology at their ends, we found that even linear plasmid DNA was recruited into the concatemer by homologous recombination.

DNA-mediated gene transfer permits the introduction of new genetic information into cultured mammalian cells. This technique is useful for studying the factors which influence gene expression, studying the mechanism of somatic cell recombination, and isolating mammalian genes. In 1977, Bacchetti and Graham (3), Maitland and McDougall (23), and Wigler et al. (41) demonstrated that the herpes simplex virus thymidine kinase gene (HSV-tk gene) could be transferred into LMtk$^-$ cells, a mouse cell line deficient in thymidine kinase. The rare transformants that expressed thymidine kinase activity were isolated by growing the cells in HAT medium (36). Cellular uptake of the purified DNA was facilitated by the formation of a DNA-calcium phosphate coprecipitate (18). Coprecipitation of the selectable gene with carrier DNA was found to increase the transformation efficiency by two to three orders of magnitude. Although the role of the carrier DNA, which can be any vertebrate DNA, has not been fully elucidated, this DNA may provide sequences or DNA structures which enhance integration.

Nonselectable genes can also be introduced into cultured mammalian cells by cotransformation with unlinked but selectable genes (42). By this method virtually any purified gene can be introduced into mammalian cells. Perucho et al. (28) have shown that cotransformed sequences are linked in transformed cells such that loss of the selectable gene from the transformant is frequently accompanied by a concurrent loss of all cotransformed sequences. Perucho et al. also demonstrated that fragments of carrier DNA and DNA for selectable and nonselectable genes are randomly ligated together in the recipient cells to form a large concatenate, which can exceed

0.2% of the haploid genome of the host. The concatenate is integrated into one or a very few sites in the host chromosome to form a stable transformant (30, 32). In independent stable transformants the concatenate is integrated into different chromosomes (30). In contrast, in unstable transformants the concatenate may exist transiently as an independent replicating unit (31).

An alternative method for transferring purified genes into cultured mammalian cells is to inject DNA into nuclei with glass micropipettes (2, 9, 17). The conditions for gene transfer by microinjection and calcium phosphate coprecipitation differ in two important ways. Microinjections are done in the absence of carrier DNA, and the amount of DNA introduced can be controlled more precisely. It has been shown that in the absence of carrier DNA, the injection of only a few molecules per cell is sufficient to obtain transformants with a high efficiency (9). The molecular events that affect a few plasmid DNA molecules introduced into the nuclear environment may be quite different from the events faced by the same plasmid DNA molecules introduced as a calcium phosphate coprecipitate in a sea of carrier DNA.

In this study we examined the fate of DNA introduced into cultured mammalian cells by microinjection.

MATERIALS AND METHODS

The methods used for culturing cells, autoradiography, plasmid DNA preparation, and microinjection have been described elsewhere (9, 10).

Southern transfer analysis. Confluent plates of cells were rinsed with phosphate-buffered saline and lysed by adding 3 ml of lysis buffer (0.5% sodium dodecyl sulfate, 100 mM NaCl, 10 mM EDTA, 20 mM Tris, pH 7.6) to each 100-mm plate. Pronase was added to a final concentration of 0.2 mg/ml, and the lysates were incubated at 37°C for 1 to 2 h. The lysates were extracted with phenol-chloroform (1:1) and chloroform before precipitation with 2 volumes of ethanol. The DNA was washed with 70% ethanol, air dried, and suspended in 10 mM Tris (pH 7.4)–1 mM EDTA. Cellular DNAs were cleaved to completion with restriction enzymes according to the instructions of the manufacturers (New England Biolabs and Bethesda Research Laboratories). The extent of digestion was monitored by adding λ DNA as an internal standard. The cleaved cellular DNA (10 to 20 μg/well) was subjected to electrophoresis through neutral 0.6% agarose gels and transferred to nitrocellulose filters by the method of Southern (33). Plasmid sequences were detected by hybridization with the appropriate recombinant tk gene-containing plasmid labeled with ^{32}P by nick translation (24, 29) with [α-^{32}P]dCTP to a specific activity of $>10^8$ dpm/μg. After prehybridization, the filters were hybridized in 4× SET (150 mM NaCl, 2 mM EDTA, 30 mM Tris [pH 8]) containing 0.1% sodium dodecyl sulfate, 0.1% sodium pyrophosphate, 20 μg of tRNA per ml, and 10× Denhardt solution (0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrollidone) at a probe concentration of 10 ng/ml at 68°C for 24 to 48 h. The filters were washed stepwise in decreasing concentrations of SET buffer (3×, 1×, and 0.5× SET supplemented with 0.1% sodium dodecyl sulfate and 0.1% sodium pyrophosphate) at 68°C. The filters were then dried and exposed for 24 to 72 h at −70°C to Kodak XAR-5 film, using a Du Pont Lightning Plus intensifying screen.

In situ hybridization. Transformants were grown in T-75 flasks in Dulbecco modified Eagle medium containing HAT (0.1 mM hypoxanthine, 1.1×10^{-3} mM amethopterin, 1.65×10^{-2} mM thymidine) and 10% fetal calf serum. The cells were diluted 1:4 the night before metaphase spreads were prepared as described previously (40) from cells treated with 100 μg of colcemid per ml for 30 to 45 min at 37°C. Probe prepared by nick translation with [^3H]dTTP (>100 Ci/mol) was found to be equivalent to that prepared with [^{125}I]dCTP.

RESULTS

Parameters affecting the frequency of transformation. When plasmid DNA containing the HSV-tk gene is injected into the nuclei of LMtk$^-$ cells, some of the cells are transformed to the TK$^+$ phenotype (2, 9). The proportion of LMtk$^-$ cells that are transformed depends on the nature of the plasmid DNA into which the HSV-tk gene is inserted (9) (Table 1). Approximately 1 LMtk$^-$ cell in 100 receiving a nuclear injection of plasmid pBR322/HSV-tk was transformed to TK$^+$ (Table 1). The transformation frequency increased to more than one in five when the injected HSV-tk gene-containing plasmid also contained sequences near the simian virus 40 (SV40) origin of DNA replication (*ori*) or sequences in the long terminal repeat (LTR) of avian sarcoma virus (ASV) provirus (Fig. 1 shows recombinant plasmid construction). It is interesting that placing these SV40 or ASV "enhancer" sequences either 5′ or 3′ to the HSV-tk gene resulted in the same relative increase in transformation frequency (Table 1). The phenomenon of enhancement of DNA-mediated transformation with respect to the location and function of enhancer sequences was explored in a separate communication (P. A. Lucius, J. M. Bishop, H. E. Varmus, and M. R. Capecchi, submitted for publication).

The effect of enhancer sequences on the transformation frequency was not restricted to LMtk$^-$ cells since a comparable increase in transformation frequency was mediated by the same enhancer sequences in RAT-2tk$^-$ cells (Table 1). Interestingly, the DNA-mediated transformation efficiency of RAT-2tk$^-$ cells was consistently lower than that of LMtk$^-$ cells, even though the viability and the ability to express the HSV-tk gene transiently are nearly 100% for both cell lines.

Another factor which influences transformation efficiency is the physical form of the inject-

TABLE 1. Transformation frequencies obtained by injecting HSV-tk gene-containing plasmid DNAs[a]

Cell line	DNA injected	No. of transformants per 10^3 cells receiving an injection
LMtk$^-$	pBR322/tk	14
	pBR322/SV-0/tk A	310
	pBR322/SV-0/tk B	262
	pBR322/ASV-2LTR/tk A	220
	pBR322/ASV-2LTR/tk B	214
RAT-2tk$^-$	pBR322/tk	1
	pBR322/SV-0/tk A	48
	pBR322/SV-0/tk B	36
	pBR322/ASV-2LTR/tk A	30
	pBR322/ASV-2LTR/tk B	28

[a] LMtk$^-$ and RAT-2tk$^-$ cells were grown on cover slips (10 by 10 mm) in 35-mm petri dishes. From 1 to 50 cells per dish received nuclear injections with the DNA solutions. The number of cells per dish receiving an injection was chosen such that at the conclusion of the experiment many of the petri dishes contained no transformed colonies. After the injections the cells were incubated for 24 h in nonselective medium at 37°C in a 5% CO_2 incubator and then switched to HAT medium. After 2 weeks the dishes were scored for the presence of large, healthy colonies. We previously reported that 1 LMtk$^-$ cell in 500 receiving an injection of pBR322/HSV-tk DNA was transformed to the TK$^+$ phenotype. The higher transformation frequencies observed were attributed to improved microinjection procedures. RAT-2tk$^-$ cells were obtained from W. Topp (38).

ed plasmid DNA. As Fig. 2 shows, comparable transformation frequencies were observed by injecting more than 50 molecules of either linear or supercoiled pBR322/SV-0/tk DNA per cell. However, as the number of plasmid DNA molecules injected per cell was decreased to less than 50, a marked difference in transformation efficiency appeared. A high transformation efficiency (approximately 20%) was retained by injecting an average of as few as five linear molecules per cell, whereas the transformation frequency dropped to less than 1 in 200 when 5 to 10 supercoiled molecules were injected per cell.

The observed differences in the transforming efficiencies of linear and supercoiled molecules were not restricted to transformation of LMtk$^-$ cells with HSV-tk gene-containing vectors. Similar results were obtained when linear and supercoiled HSV-tk gene-containing plasmids were injected into RAT-2tk$^-$ cells. Moreover, we found that linear plasmids containing the selectable *Escherichia coli* gene for xanthine guanine phosphoribosyl transferase (27) gave a 30- to 50-fold-higher transformation frequency than supercoiled plasmids, when less than 10 copies per cell were injected into LMtk$^-$ or RAT-2tk$^-$ cells.

Transformants containing a single copy of the HSV-tk gene. Transformants obtained after injecting only a few plasmid molecules per cell often contained a single copy of the transforming sequence. Figure 3 shows a Southern genomic transfer analysis of transformants obtained by injecting a few copies of either supercoiled or linear plasmid DNA. Both the intensity and the patterns of hybridization signals were consistent with the conclusion that these transformants contained single copies of their respective plasmid DNAs. When genomic DNA was incubated with a restriction endonuclease that did not cleave the appropriate plasmid DNA or cleaved it only once, the respective patterns consisted of a band that migrated more slowly than intact plasmid DNA or two bands of variable size. The diversity of band sizes generated by the restriction endonucleases suggests that the transforming DNA was flanked by different nonplasmid sequences in each transformant. Using a combination of appropriate restriction enzymes, only some of which are shown in Fig. 3, we constructed a restriction map of how each plasmid molecule was integrated into host DNA sequences (Fig. 3). From such analyses there did not appear to be a preferred site of integration within the supercoiled plasmids. For example, in the three transformants shown in Fig. 3, which were obtained by injecting pBR322/SV-0/tk B or pBR322/ASV-2LTR/tk A or B supercoiled DNA, two of the plasmids were integrated into host DNA through their pBR322 sequences and the third was integrated through its ASV-2LTR leader sequence.

On the other hand, single linear molecules appeared to be inserted into the host genome through their ends (Fig. 3, lanes j through m). In the process of insertion, the restriction sites at the ends of the plasmid molecule was lost (Fig. 3, lane j). However, extensive modification at the ends of the linear molecules did not appear to be the rule. For example, in transformants obtained by injecting pBR322/ASV-2LTR/tk A linearized with *Hin*dIII, the *Cla*I site which was only 6 nucleotide base pairs away from the *Hin*dIII site was still present (Fig. 3, lane l).

Transformants containing multiple copies of the HSV-tk gene. When the recipient cells received injections of more than 5 linear or 25 supercoiled HSV-tk gene-containing plasmid DNA molecules per cell, the resulting transformants contained multiple copies of the transforming DNA. Figure 4, lanes a through c show Southern genomic transfers of independent transformants obtained by injecting approximately 25 copies of pBR322/ASV-2LTR/tk B supercoiled DNA per cell into LMtk$^-$ cells. The genomic DNA was hydrolyzed with *Bgl*II, which cut the plasmid DNA once. In these

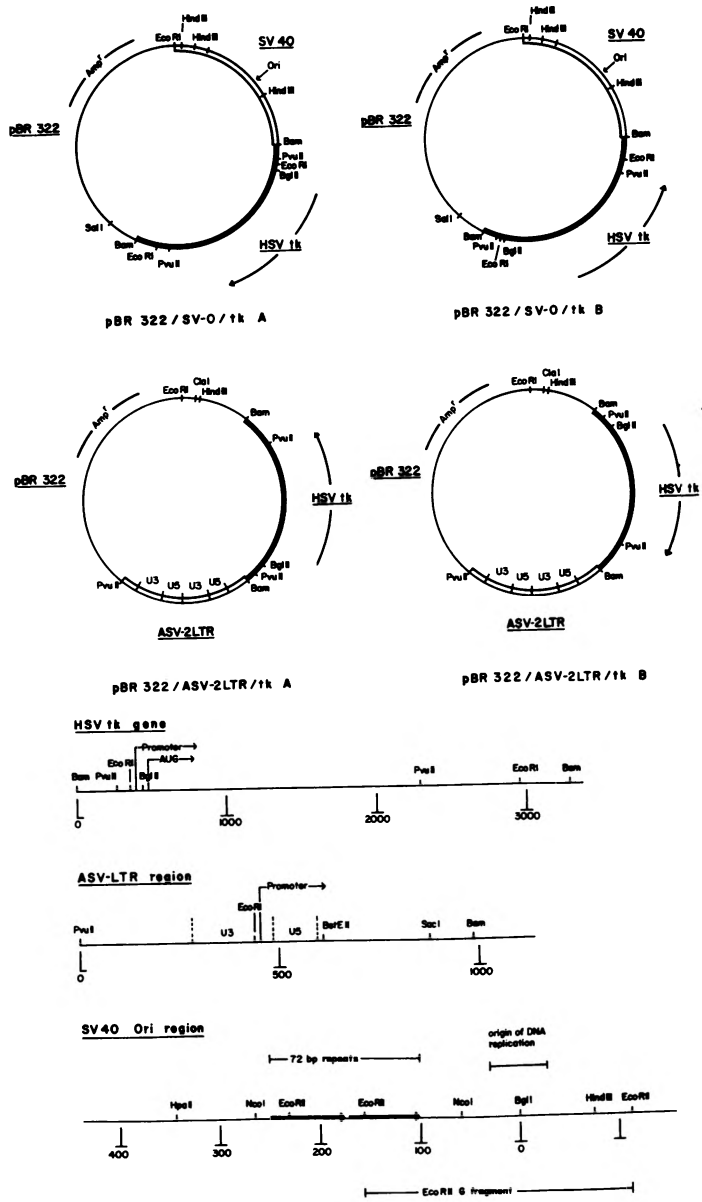

FIG. 1. Restriction maps of recombinant plasmids pBR322/SV-0/tk A, pBR322/SV-0/tk B, pBR322/ASV-2LTR/tk A, and pBR322/ASV-2LTR/tk B, as well as schematic representations of the HSV-tk gene-containing *Bam*HI (Bam) fragment, ASV LTR region, and SV40 origin of DNA replication (Ori) region. The construction of pBR322/SV-0/tk A and B recombinant plasmids has been described previously (9). pBR322/ASV-2LTR/tk A and B (obtained from and prepared by P. Luciw) were prepared by inserting the *Pvu*II fragment containing two copies of the u3u5-terminal repeats from the Schmidt-Ruppin A-2 strain of ASV DNA into pBR322 at the *Pvu*II site (14). The recombinant plasmid described above was then hydrolyzed with *Bam*HI, and the 3.4-kb *Bam*HI fragment containing the HSV-tk gene was inserted. In the process of inserting the HSV-tk gene-containing *Bam*HI fragment, 320 bp from the ASV leader sequence and 1.8 kb of the pBR322 sequence were removed. Amp^r, Ampicillin resistance.

transformants, we observed a single major band that hybridized with a ^{32}P-labeled nick-translated pBR322/ASV-2LTR/tk B probe and comigrated with intact linear pBR322/ASV-2LTR/tk B plasmid DNA. The intensity of this band relative to bands in single-copy transformants was consistent with the conclusion that these transformants contained 10 to 20 copies of the plasmid sequences. We often observed two other bands, which hybridized less intensely and the sizes of

which varied from transformant to transformant. These minor bands probably represented the fragments which included the junction between the concatemer and host sequences. When DNAs from the transformants described above were digested with an endonuclease that did not cleave the plasmid DNA, hybridization occurred over a single band that migrated much more slowly than linear plasmid DNA (data not shown). The above-described results are only consistent with the conclusion that the multiple plasmid sequences were present in the transformants as head-to-tail concatemers, since a random arrangement of molecules within the concatemers would have yielded multiple bands after cleavage with *Bgl*II.

In Fig. 4, lanes d through g, we show a series of LMtk$^-$ and RAT-2tk$^-$ transformants obtained by injecting approximately 25 copies of pBR322/ASV-2LTR/tk A DNA linearized with *Hin*dIII per cell. The patterns shown by a Southern transfer analysis of the genomic DNAs from these transformants digested with *Bgl*II were indistinguishable from the patterns obtained by injecting pBR322/ASV-2LTR/tk A or B supercoiled molecules. This result was surprising and indicated that the multiple copies of the HSV-tk gene-containing plasmid injected as linear molecules were incorporated into head-to-tail concatemers. To illustrate the expected hybridization pattern if the multiple gene copies within the concatemer were randomly oriented, pBR322/ASV-2LTR/tk A DNA was linearized with *Hin*dIII, ligated in vitro to form a random concatemer, digested with *Bgl*II, and electrophoresed in parallel with the genomic DNA digests. Three bands, corresponding to adjacent DNA molecules that were linked in the head-to-head (8.9 kilobases [kb]), head-to-tail (7.4 kb), and tail-to-tail (6.0 kb) configurations, were observed (Fig. 4, lane h). In some of the transformants obtained by injecting linear molecules, a hint of the bands predicted for adjacent molecules that were oriented head to head or tail to tail was observed. However, the relative intensities of these bands were not what would be predicted for a random concatemer (1:2:1 for the head-to-head, head-to-tail, and tail-to-tail configurations, respectively).

Genomic DNAs from several of these transformants were hydrolyzed with *Hin*dIII and analyzed as described above to determine how often the *Hin*dIII site was preserved during the incorporation of the linear plasmid DNA into the head-to-tail concatemer. Loss of a single nucleotide base pair at the ends during concatemer formation would have resulted in the loss of the *Hin*dIII site. This loss was revealed on the Southern transfer as a band that comigrated with a linear plasmid dimer or higher multimer. More than 90% of the *Hin*dIII sites were preserved

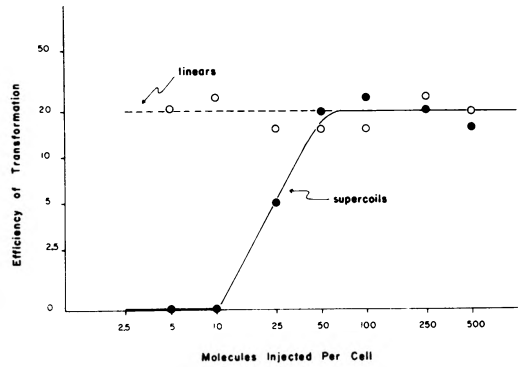

FIG. 2. Comparison of the frequency of transforming LMtk$^-$ to the TK$^+$ phenotype after nuclear injections of increasing amounts of linear (○) and supercoiled (●) pBR322/SV-0/tk B DNAs. The experimental procedure was as described in Table 1, footnote *a*. pBR322/SV-0/tk B plasmid DNA was linearized with *Sal*I.

during concatemer formation, indicating that the plasmid DNA underwent very little damage at the ends of the linear molecule before or during concatemer formation (data not shown; however, a similar conclusion can be reached from the experiments shown in Fig. 7, lanes i through l).

Head-to-tail concatemers are integrated into the host chromosome. Several transformants were examined by hybridization in situ to determine whether the transforming sequences were indeed integrated into host chromosomes. Two of the transformants chosen for this study, L11 and L278, were shown by a Southern transfer analysis to contain approximately 10 copies of pBR322/SV-0/tk B and 30 copies of pBR322/ASV-2LTR/tk B, respectively, as head-to-tail concatemers. L11 and L278 revealed a single pair and two pairs of junction fragments, respectively, which is consistent with integration of these concatemers at one and two sites in the host genome, respectively. Figure 5 shows metaphase spreads of L11 and L278 hybridized in situ to HSV-tk gene-containing recombinant plasmid DNA labeled by nick translation with [^{125}I]dCTP or [^{3}H]dTTP. The transforming DNA in L11 could be localized to a single chromosome, whereas in L278 the plasmid sequences appeared to have been integrated into two sites. Hybridization of the plasmid probe to parental LMtk$^-$ chromosomes showed no labeling (data not shown). The site of integration was different for other transformants examined.

How are the head-to-tail concatemers generated? Figure 6 shows three models by which head-to-tail concatemers could be generated. In model I a single plasmid sequence is first integrated into host DNA and then amplified, generating a head-to-tail concatemer. In models II and III the

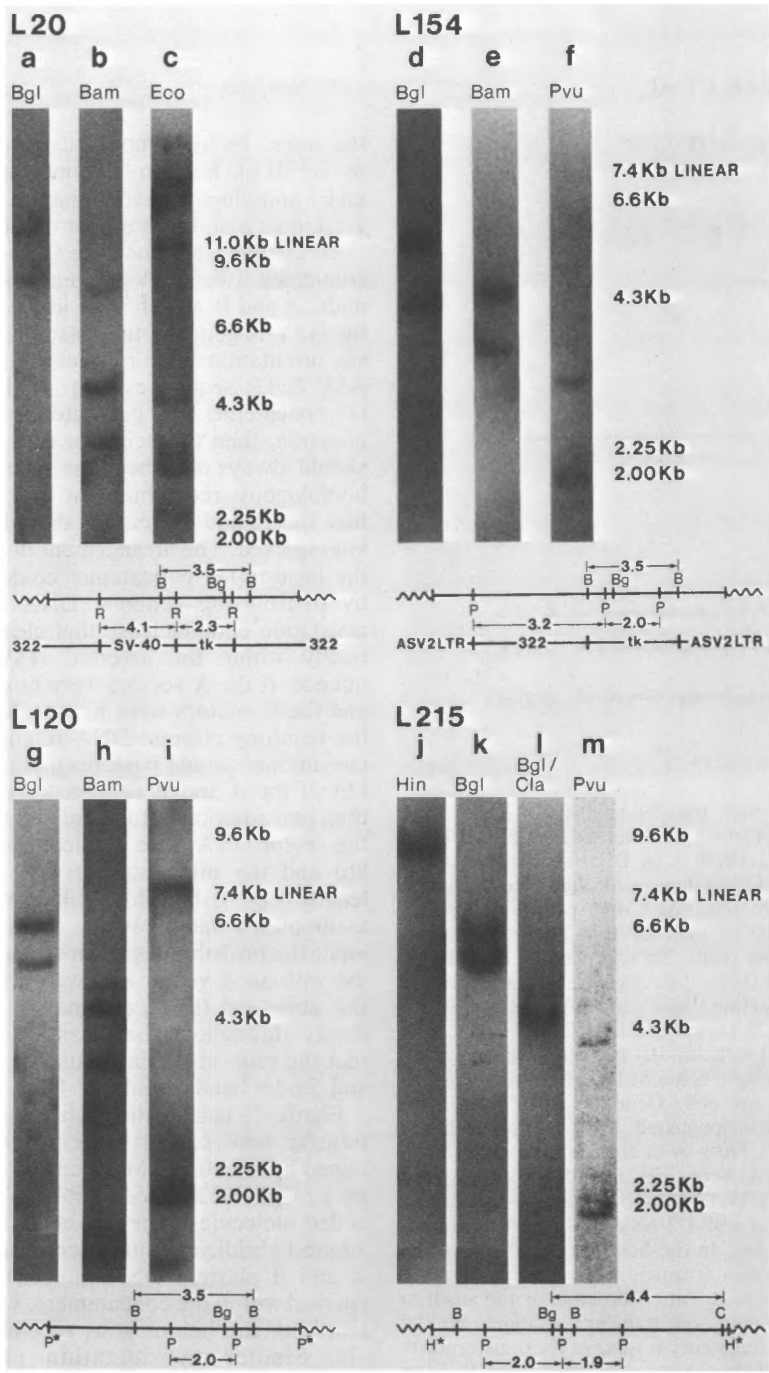

FIG. 3. Southern transfer analysis of LMtk$^-$ transformants containing single copies of the HSV-tk gene-containing vectors. Transformants L20 (lanes a through c), L154 (lanes d through f), and L120 (lanes g through i) were obtained by injecting LMtk$^-$ cells with approximately 10 copies of supercoils of pBR322/SV-0/tk B, pBR322/ASV-2LTR/tk B, and pBR322/ASV-2LTR/tk A DNAs per cell, respectively. L215 (lanes j through m) was obtained by injecting LMtk$^-$ cells with less than five copies of pBR322/ASV-2LTR/tk A DNA linearized with *Hin*dIII per cell. The restriction maps of the HSV-tk gene-containing vectors inserted into host DNA sequences were deduced from the Southern transfer data in lanes a through m. These maps were also consistent with a Southern transfer analysis of genomic DNAs digested with *Bam*HI, *Cla*I, *Hin*dIII, *Sal*I, *Cla*I plus *Bgl*II, *Cla*I plus *Bam*HI, and *Cla*I plus *Bgl*II plus *Bam*HI (data not shown). B and Bam, *Bam*HI; Bg and Bgl, *Bgl*II; C and Cla, *Cla*I; H and Hin, *Hin*dIII; P and Pvu, *Pvu*II; R and Eco, *Eco*RI. H* and P* indicate that the *Hin*dIII and *Pvu*II plasmid restriction sites were lost as a result of the plasmid DNA being inserted into the host genome. The extra band in the *Eco*RI digest of L20 (lane c) was a result of partial cleavage of the genomic DNA. In the *Bgl*II digest of L215 there are two bands, at 6.6 and 6.0 kb; in the *Bgl*II-*Cla*I digest there is a single band, at 4.4 kb. This indicates that a *Cla*I site is present in the flanking host DNA approximately 4.4 kb upstream from the *Bgl*II site and that the 4.4-kb band is a doublet. Consistent with this interpretation, we observed a band of approximately 9 kb when L215 DNA was cleaved with *Cla*I alone.

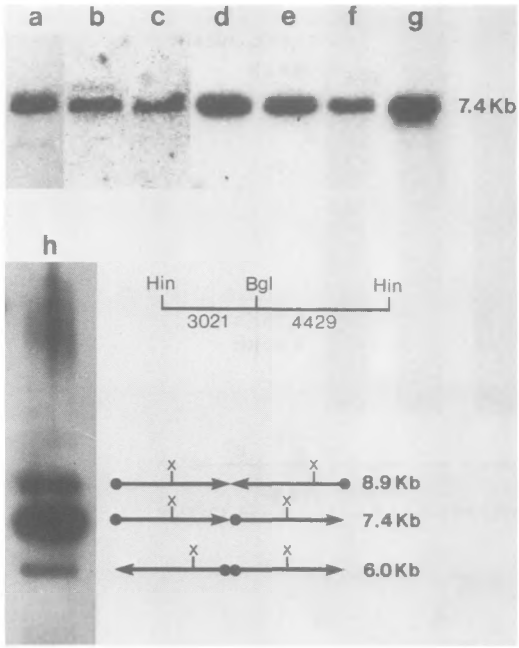

FIG. 4. Southern transfer analysis of LMtk$^-$ and RAT-2tk$^-$ transformants containing multiple copies of pBR322/ASV-2LTR/tk A or B DNA. Transformants L161, L162, and L163 (lanes a through c, respectively) were obtained by injecting LMtk$^-$ cells with approximately 25 copies of pBR322/ASV-2LTR/tk B supercoiled DNA per cell. Transformants L211, L212, R211, and R221 (lanes d through g, respectively) were obtained by injecting LMtk$^-$ and RAT-2tk$^-$ cells with approximately 25 copies of pBR322/ASV-2LTR/tk A DNA linearized with *Hin*dIII per cell. The transformants described above contained from 10 to 25 copies of plasmid DNA per cell. Genomic DNA from each transformant was incubated with *Bgl*II, which cut pBR322/ASV-2LTR/tk A or B once. The major band that hybridized with ^{32}P-labeled nick-translated pBR322/ASV-2LTR/tk A or B sequences comigrated with intact linear pBR322/ASV-2LTR/tk A or B plasmid DNA (7.4 kb). In the Southern transfers we did not detect junction fragments, presumably because these fragments were either too large or too small to allow detection under our transfer conditions. We did detect junction fragments in some of the transformants after cleavage of the genomic DNA with *Bam*HI. Lane h shows the Southern transfer pattern obtained by hydrolyzing with *Bgl*II a concatemer synthesized in vitro in which the pBR322/ASV-2LTR/tk A sequences were randomly oriented (see text). Hin, *Hin*dIII; Bgl, *Bgl*II.

head-to-tail concatemers are generated by a mechanism involving homologous recombination. In model II a single plasmid molecule is integrated into the host chromosome, perhaps by a nonhomologous recombination event, and subsequent plasmid molecules then integrate at the same site by homologous recombination. In model III the head-to-tail concatemer is generated by homologous recombination before its integration as a unit into a host chromosome.

To distinguish among the various models, we coinjected two HSV-tk gene-containing plasmids, A and B, which were identical except that the HSV-tk genes of the plasmids were in opposite orientations with respect to the pBR322 and ASV-2LTR sequences (Fig. 1). If the head-to-tail concatemer was generated by sequence duplication, then the neighbor of a given plasmid should always have been the same plasmid. The homologous recombination models predicted that the A and B vectors should be randomly interspersed. The arrangement of vectors within the head-to-tail concatemer could be identified by hydrolyzing genomic DNA with *Bgl*II, a restriction endonuclease that cleaves asymmetrically within the inverted HSV-tk gene sequence. If the A vectors were next to A vectors and the B vectors were next to B vectors, then the resulting plasmid DNA fragments from the concatemer should have been of unit length (7.4 kb). If the A and B vectors were interspersed, then two additional fragments that hybridized to the vector DNA were predicted, one longer (9.3 kb) and the other shorter (5.6 kb) than unit length (Fig. 7, lane h). Making the reasonable assumptions that (i) under conditions of equal input the probabilities of an A vector recombining with an A vector and with a B vector were the same and (ii) recombination occurred randomly throughout the plasmids, we predicted that the ratio of the intensities of the 9.3-, 7.4-, and 5.6-kb bands would be 1:6:1.

Figure 7, lanes a through c, show Southern transfer analyses of three transformants obtained by injecting LMtk$^-$ cells with equal numbers of pBR322/ASV-2LTR/tk A and B supercoiled molecules. The presence of the 9.3- and 5.6-kb hybridizing bands demonstrated that the A and B plasmid DNA molecules were interspersed within the concatemers, which is consistent with the homologous recombination models. Similar hybridization patterns were observed with transformants generated by coinjecting equal numbers of linear A and B molecules (Fig. 7, lanes d through g). As a reference for random end-to-end ligation, the A and B vectors were linearized with *Hin*dIII, mixed, ligated in vitro, and hydrolyzed with *Bgl*II. The more complex but predictable hybridization pattern obtained is shown in Fig. 7, lane h. Our results indicate that even with linear molecules, the concatemers were formed predominantly by homologous recombination rather than by random end-to-end ligation or amplification.

Deviation of the relative intensities of the 9.3-, 7.4-, and 5.6-kb bands in some of the transfor-

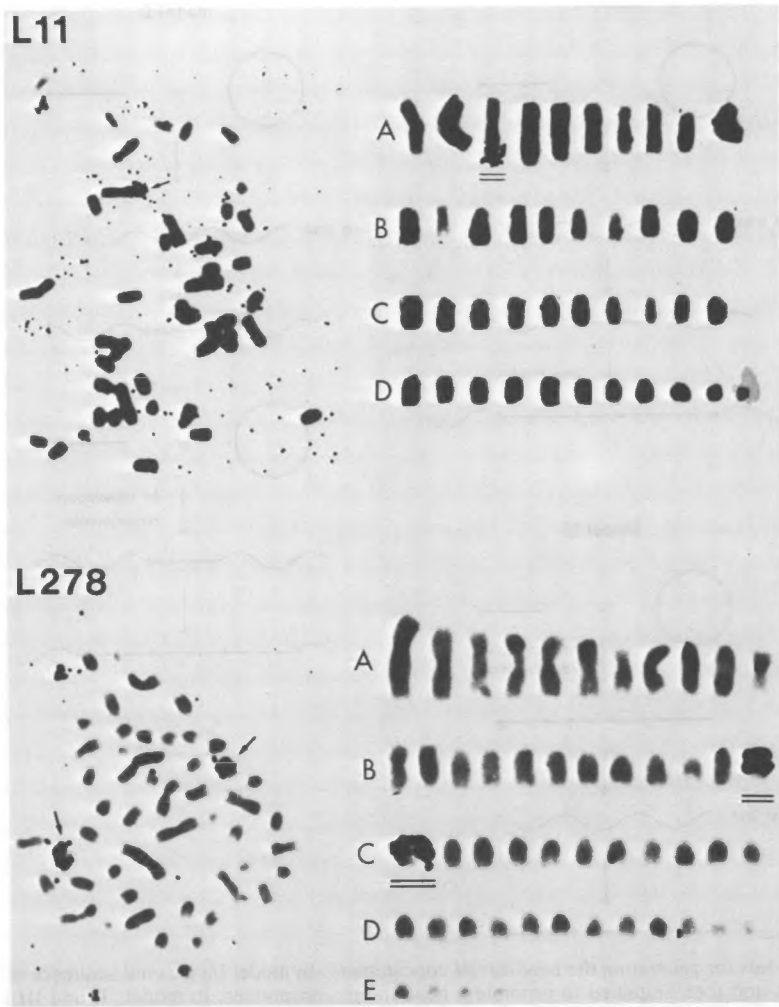

FIG. 5. In situ localization of the donated genes in transformants L11 and L278. Metaphase spreads of L11 and L278 were prepared as described in the text and hybridized with probes made by nick translation of recombinant HSV-tk gene-containing plasmids with either [^{125}I]dCTP (L11) or [^{3}H]dTTP (L278) to a specific activity of $\sim 5 \times 10^7$ cpm/μg. Each slide was hybridized overnight with 2.5×10^5 cpm of probe in a total volume of 25 μl in the presence of 10% sodium dextran sulfate 500. The slides were washed and exposed at 4°C for approximately 2 weeks. The left side of the figure shows the spreads from which the chromosomes on the right side were excised; these chromosomes are arranged according to size. Significant hybridization to the indicated chromosome of L11 (row A) was observed in six of six metaphase preparations examined. For L278, 20 of 25 metaphase preparations showed the chromosome in row B, and 15 of 25 showed the chromosome in row C. In spreads with fewer grains these chromosomes were clearly of different sizes.

mants from the predicted 1:6:1 ratio may have reflected stochastic fluctuations from randomness in the interspersion patterns of the A and B vectors within the concatemers. Such fluctuations were anticipated since these transformants did not contain large numbers of plasmid DNA copies. For example, a further analysis of the transformant shown in Fig. 7, lane a, indicated that this transformant contained three copies of the pBR322/ASV-2LTR/tk vectors in the arrangement A-A-B. Alternatively, the deviation in the ratio may have reflected more complex mechanisms for generating the head-to-tail concatemers, which would have included combinations of homologous recombination and amplification mechanisms.

We estimated the relative frequency with which homologous recombination occurred within the HSV-tk gene compared with the pBR322/ASV-2LTR sequences by performing the following analysis. Genomic DNA was cleaved with *Hin*dIII, which cut asymmetrically within the pBR322/2LTR sequences, or with *Bgl*II, which cut asymmetrically within the

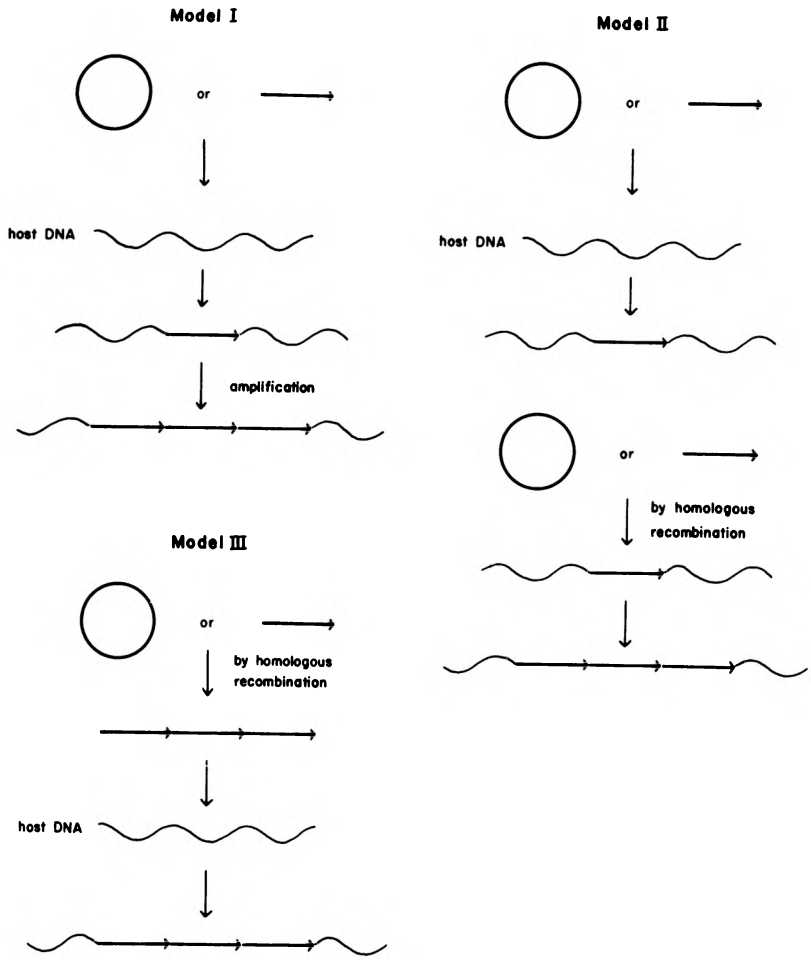

FIG. 6. Models for generating the head-to-tail concatemers. In model I a plasmid sequence is first integrated into host DNA and then amplified to generate a head-to-tail concatemer. In models II and III the head-to-tail concatemers are generated by a mechanism involving homologous recombination. In model II a single plasmid molecule is integrated into the host chromosome, and subsequent plasmid molecules then integrate at the same site by homologous recombination to generate the head-to-tail concatemer. In model III the head-to-tail concatemer is generated by homologous recombination before its integration as a unit into the host chromosome.

HSV-tk gene sequences. Such cleavages generated unique hybridization patterns depending on the site of recombination. If homologous recombination between A and B molecules occurred within the HSV-tk gene, then cutting with *Bgl*II resulted in a fragment of unit length, but cutting with a restriction enzyme that cut in the pBR322/ASV-2LTR sequence (*Hin*dIII) generated fragments of nonunit length (10.7 and 4.2 kb). Similarly, if homologous recombination occurred within the pBR322/ASV-2LTR sequence, cutting with *Hin*dIII resulted in a fragment of unit length, whereas cutting with *Bgl*II resulted in fragments of nonunit length (9.3 and 5.6 kb). Figure 7, lanes i through l show Southern transfers of *Hin*dIII digests of the same transformants shown in Fig. 7, lanes d through g (i.e., *Bgl*II digests). In addition to the major hybridizing band of unit length (7.4 kb) we also observed bands at 10.7 and 4.2 kb. Thus, recombination appeared to have occurred in both the HSV and the pBR322/ASV-2LTR sequences. Similar results were obtained when supercoiled or linear A and B vectors were coinjected. With transformant L351 (Fig. 7, lanes d and i) recombination occurred with equal frequency in the HSV and pBR322/ASV-2LTR sequences. With transformants L352, L353, and L354, we observed a bias toward more recombination in the pBR322/ASV-2LTR region of the plasmid. This bias may reflect stochastic fluctuations within the small sample size or preferential sites of recombination.

In two of the *Hin*dIII digests (Fig. 7, lanes k and l) we also observed a plasmid dimer band (14.9 kb). This band could have arisen either

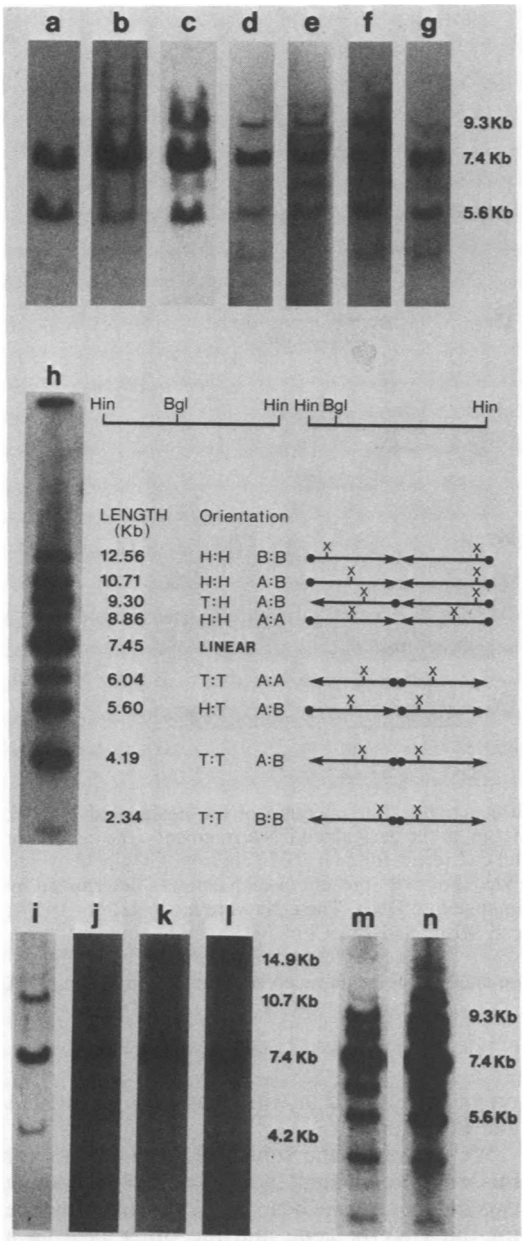

FIG. 7. Southern transfer analysis of transformants obtained by coinjecting pBR322/ASV-2LTR/tk A and B DNAs. Transformants L301, L302, and L303 (lanes a through c, respectively) were obtained by coinjecting 25 copies of both pBR322/ASV-2LTR/tk A and B supercoiled DNAs. Genomic DNAs from these transformants were hydrolyzed with *Bgl*II and processed by the method of Southern (33). In addition to the major hybridizing band of unit length (7.4 kb), we also observed bands which were 9.3 and 5.6 kb long. These latter bands arose from adjacent A and B vectors that recombined in the pBR322/ASV-2LTR sequences (see text). Transformants L351, L352, L353, and L354 (lanes d through g, respectively) were obtained by coinjecting approximately 15 copies of both pBR322/ASV-2LTR/tk A and B linear DNAs (linearized with *Hin*dIII). Genomic DNAs from these transformants were hydrolyzed with *Bgl*II. The hybridization patterns are very similar to those obtained by coinjecting A and B supercoiled DNAs. Lane h shows the Southern transfer pattern of a *Bgl*II digest of an in vitro synthesized concatemer in which the A and B vectors were randomly oriented (see text). A schematic diagram for the origin of each *Bgl*II fragment is provided. Lanes i through l show the Southern transfer patterns of *Hin*dIII digests of genomic DNA from transformants L351, L352, L353, and L354, respectively (see above). In addition to the major hybridizing band of unit length (7.4 kb), we also observed bands which were 10.7 and 4.2 kb long. These bands arose from adjacent A and B vectors that recombined in the HSV-tk gene containing *Bam*HI sequence (see text). Transformants L356 and L357 (lanes m and n, respectively) were obtained by coinjecting 75 copies of each pBR322/ASV-2LTR/tk A and B linear DNA. The Southern transfer pattern of the *Bgl*II digest of these transformants is similar to the marker pattern (lane h). A quantitative examination of these blots indicated that a majority of the A and B vectors within the concatemers were still oriented head to tail. Hin, *Hin*dIII; Bgl, *Bgl*II; H:H, head to head; H:T, head to tail; T:T, tail to tail.

from the loss of a *Hin*dIII site during incorporation of linear A and B molecules into the concatemer or from incomplete digestion of genomic DNA with *Hin*dIII. The ratio of monomer to dimer bands did not change after genomic DNA was digested with two- and fourfold excess concentrations of *Hin*dIII. The other two transformants (Fig. 7, lanes i and j), which were also obtained by coinjecting A and B vectors linearized with *Hin*dIII, did not exhibit plasmid dimer bands.

The results of the experiments discussed above argue strongly that the head-to-tail concatemers are generated by homologous recombination; however, these experiments did not distinguish between models II and III. Formal distinction between models II and III is indeed difficult because these two models utilize the same enzymatic machinery and differ only in the kinetic appearance of the postulated intermediates.

Stability of the head-to-tail concatemers. Since the head-to-tail concatemers appeared to be formed by homologous recombination, it was of interest to determine whether these sequences were stable after integration into the host chromosome. It was possible that these concatemers would be lost readily by a mechanism which used, for example, the same enzymatic machinery that led to their formation.

Three transformants containing head-to-tail tandem repeats were grown in nonselective medium for 50 generations. The stability of the concatemers was measured periodically by Southern transfer analysis, which estimated the

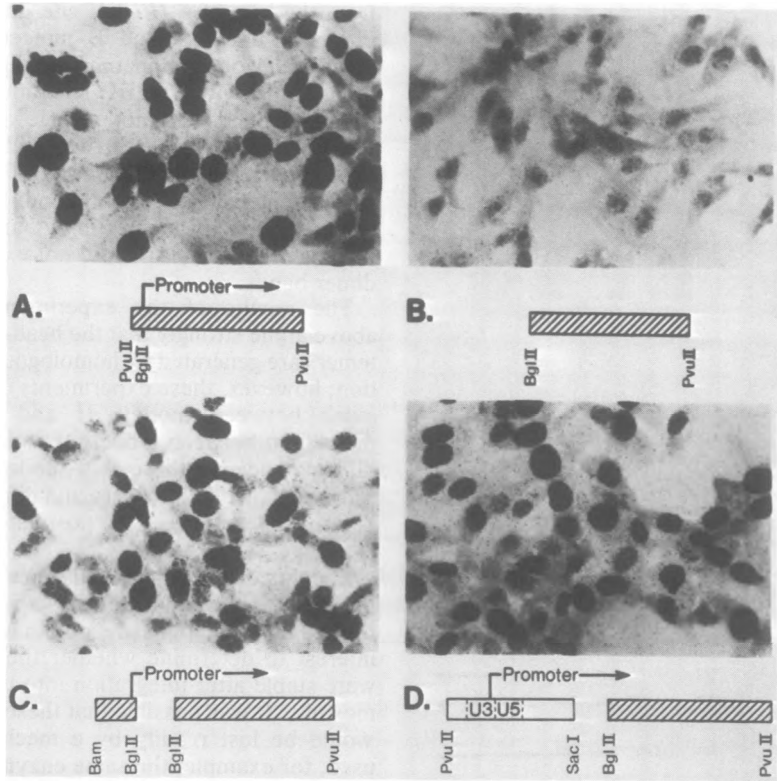

FIG. 8. Autoradiographic analysis for thymidine kinase activity after injection of the designated HSV-tk gene-containing and ASV LTR DNA fragments. After injection of the designated DNA fragments, the cells were incubated in minimal essential medium containing 10% fetal calf serum for 24 h at 37°C in a 5% CO_2 incubator to allow for the expression of HSV-tk genes. The thymidine kinase activity present in each cell was determined by measuring the capacity of the cells to incorporate [^{3}H]thymidine into DNA. The cells were incubated for 16 h at 37°C in minimal essential medium containing 10% fetal calf serum and 25 μCi of [^{3}H]thymidine per ml. After incubation in [^{3}H]thymidine-containing medium, the cells were washed, fixed, and processed for autoradiography as previously described (9). A total of 25 to 30 cells in each of the fields received an injection of the DNA fragments. Bam, *Bam*HI.

average number of plasmid DNA copies per cell, and by in vivo [^{3}H]thymidine incorporation into DNA, followed by autoradiography, which measured the fraction of cells which retained HSV-tk gene enzymatic activity. Over the duration of the experiment there was no detectable loss of plasmid sequences, and no increase in the fraction of TK^- cells was observed.

End-to-end ligation. We observed that if large quantities of linear A and B vectors (i.e., more than 75 copies of each per cell) were coinjected, then some random end-to-end ligation was detected (Fig. 7, lanes m and n). The Southern transfer pattern for these transformants was very similar to the marker pattern (Fig. 7, lane h) generated by ligation of the two vectors in vitro. A careful examination of these Southern transfers indicated that the transformants contained concatemers composed of A and B vectors which were predominantly in the head-to-tail orientation mixed in with randomly oriented A and B vectors.

We examined the substrate requirements for this end-to-end ligation in vivo by coinjecting two fragments (one bearing the coding sequence for the HSV-tk gene and the other bearing a promoter sequence) and measuring HSV-tk gene enzymatic activity (Fig. 8). Approximately 50 promoter fragments were coinjected with 25 HSV-tk gene-containing *Bgl*II-*Pvu*II fragments. We made the assumption that in order to make a functional transcript, the two fragments had to be correctly ligated in the recipient cell. Quantitation for this experiment is shown in Fig. 9, along with diagrams of the coinjected fragments. The results are presented as the ratio of the number of cells exhibiting HSV-tk gene activity to the number of cells receiving injections with the respective fragment(s). As a positive control, cells were injected with the HSV-tk gene-

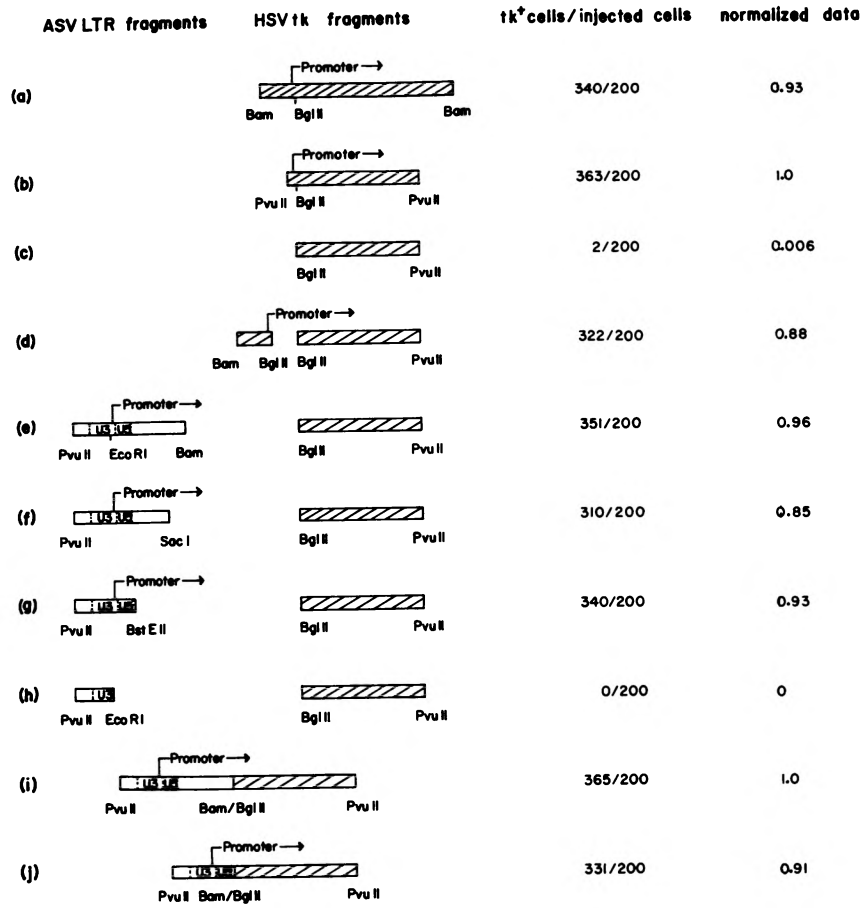

FIG. 9. Number of TK^+ cells after injection of $3T3tk^-$ cells with HSV-tk gene-containing and ASV LTR DNA fragments. The thymidine kinase activity present in each cell was determined autoradiographically as described in the legend to Fig. 8. The number of TK^+ cells could exceed the number of injected cells because the [^{3}H]thymidine incorporation assays were done 24 h after injection; by this time many of the cells had divided. Bam, *Bam*HI.

containing *Bam*HI and *Pvu*II fragments, both of which contained the intact HSV-tk gene promoter and the HSV-tk structural gene. The number of TK^+ cells exceeded the number of cells receiving an injection (Fig. 9a and b) because the [^{3}H]thymidine incorporation assays were done 24 h after injection, by which time many of the cells had divided. Very few TK^+ cells were observed among the cells that received injections of the control HSV-tk gene-containing *Bgl*II-to-*Pvu*II fragment (Fig. 8B and 9c), which lacked a functional promoter (25). Coinjection of the HSV-tk gene-containing *Bam*HI-to-*Bgl*II fragment, which contained the HSV-tk gene promoter, with the HSV-tk gene-containing *Bgl*II-to-*Pvu*II fragment resulted in many TK^+ cells, indicating that a functional ligation of the two fragments occurred (Fig. 8C and 9d).

When we began these experiments, we knew that the ASV LTR could be fused to the HSV-tk gene at the *Bgl*II site and function as an effective promoter (P. Luciw and M. R. Capecchi, unpublished data). In transformants obtained by injecting the fused gene, Luciw detected a hybrid ASV-5′–HSV-tk gene message which was initiated at or very near the ASV cap site (data not shown). The point of fusion could be at the ASV *Bam*HI or *Bst*EII site (Fig. 1 and Figs. 9i and j), indicating that the length of the leader sequence did not affect the activity of the resulting transcript.

With these results in mind, we could test whether the in vivo end-to-end ligation reaction required homologous ends. The ASV LTR fragment contains *Bam*HI, *Sac*I, and *Bst*EII sites in the leader. The *Sac*I and *Bst*EII restriction sites show no homology to the *Bgl*II site. Furthermore, *Sac*I generates a 3′ overhang, whereas *Bgl*II generates a 5′ overhang. As shown in Fig. 9e through g, ASV LTR fragments which termi-

nated at the *Bst*EII, *Sac*I, and *Bam*HI sites were all ligated efficiently to the *Bgl*II-to-*Pvu*II HSV-tk gene-containing fragment to form a functional gene. As an additional control, the ASV LTR fragment terminating at the *Eco*RI site was coinjected with the HSV-tk gene-containing *Bgl*II-to-*Pvu*II fragment. The *Eco*RI site in the ASV LTR sequence is upstream (5') of the TATA box (35). No TK^+ cells were observed in cell preparations that received injections of these two fragments (Fig. 9h). Also, no TK^+ cells were observed among cells that received injections of only promoter fragments (data not shown).

From the experiments described above, it is clear that two DNA fragments can be ligated together in mammalian nuclei irrespective of the sequence or topology at their ends. Since any two DNA fragments appear to be joined by ligation, many of the ligation reactions that occurred in the above-described experiments must have been nonfunctional (e.g., promoter fragments that were joined together, HSV-tk gene-containing *Bgl*II-*Pvu*II fragments that were joined together, promoter fragments that were joined downstream to the HSV-tk gene-containing *Bgl*II-*Pvu*II fragment, etc.). In fact, if any two fragments can be joined together, then only 1 in 12 possible ligation reactions would be expected to be productive. Since we observed a productive ligation in nearly every cell that received an injection, the efficiency of joining two free ends of any DNA molecules together is extremely high.

DISCUSSION

The ability of cultured mammalian cells to incorporate exogenous DNA into their chromosomes can be remarkably efficient when DNA is injected directly into the nucleus through glass micropipettes. We have obtained transformation efficiencies greater than 20% by injecting as few as three linear molecules per cell. As discussed below, the transformation efficiency reflects the physical form of the injected molecules, the presence of enhancer sequences, and the type of recipient cells used. The number of gene copies integrated into the genome can be varied by simply altering the quantity of DNA injected. Using this method, we have obtained transformants containing a single copy or as many as 50 to 100 copies of the donated genes. Although the number of gene copies which are stably integrated is roughly proportional to the number of gene copies injected, there is considerable variation from transformant to transformant. This fluctuation may indicate that there are periods of the cell cycle during which exogenous DNA can be incorporated into the genome more efficiently.

As demonstrated by in situ hybridization, multiple copies of the transforming DNA are integrated at one or a very few sites in the host chromosome, and the site of integration is different in independent transformants. Robins et al. (30) and de Saint Vincent et al. (15) previously reached the same conclusion for transformants obtained by calcium phosphate coprecipitation and protoplast fusion, respectively. These results suggest that a rate-limiting step in the DNA-mediated transformation process may be the insertion of exogenous DNA into a host chromosome. It will be of interest to determine whether the lower transformation efficiency of RAT-$2tk^-$ cells can be correlated to this rate-limiting step.

In more than 100 transformants we have never found free plasmid DNA as supercoiled, linear, or relaxed circular molecules. Such molecules would have been detected by Southern transfer analysis of either undigested genomic DNA or genomic DNA digested with a restriction enzyme that does not cut the plasmid DNA.

We have investigated the process by which the donated molecules become integrated into the host chromosome by varying the numbers and structures of the injected molecules. When the cell nucleus is presented with supercoiled molecules, no preferred site of integration with respect to plasmid sequences is observed in transformants containing a single copy of the plasmid. Single linear molecules, on the other hand, appear to be inserted into the host genome through their ends. In the process of insertion, the restriction sites at the ends of the linear molecule are lost, but a site as few as six nucleotides from the terminus is found intact.

A number of other observations also indicate that the DNA injected into nuclei of $LMtk^-$ or RAT-$2tk^-$ cells is not extensively hydrolyzed or rearranged before or during integration into the host chromosome. Within the resolution of the Southern transfer technique, analyses of a number of single-copy transformants have indicated that the sum of the lengths of internal fragments is equal to the length of the intact plasmid. Furthermore, DNA from a number of transformants containing multiple copies of the plasmid vector have been analyzed by digestion with a series of restriction enzymes. Aside from the expected junction fragments, we have not frequently observed spurious hybridizing bands that cannot be accounted for by restriction maps of the vectors. Also, in transformants obtained by injecting multiple copies of a plasmid DNA linearized with a particular restriction enzyme, the loss of the restriction site of the enzyme in the concatemer is less than 10%.

When more than 5 linear or 25 supercoiled molecules are injected per cell, the transformants contain multiple copies of the donated sequences in a head-to-tail array. By analyzing

transformants obtained by coinjecting two vectors which are identical except that in one a portion of the vector sequence is inverted, we have been able to conclude that the head-to-tail concatemers are generated predominantly by homologous recombination and that recombination can occur throughout the vector.

The interspersion of the A and B vectors in the same concatemer demonstrates that homologous recombination can account for the formation of the head-to-tail concatemers but does not rule out the possibility that amplification is a contributing mechanism. However, the additional observation that the number of gene copies integrated into the host chromosome is roughly proportional to the number of gene copies injected into the recipient cells argues against frequent amplification of the donated DNA sequences. Furthermore, we have not observed transformants containing many copies of the donated plasmid sequences after injection of only a few copies of the plasmid DNA.

Head-to-tail concatemers of papovavirus DNA have been observed after transfection of nonpermissive cells with SV40 or polyoma virus DNA (4, 7, 12, 13, 26). Replicative synthesis of the viral DNA has been implicated as the mechanism for generating these concatemers. Our results differ from the results of other workers (4, 7, 12, 13, 26) in that the plasmid sequences which we injected were presumed to be replicatively incompetent.

It is interesting that when small numbers of plasmid molecules are injected into the recipient, linear molecules transform LMtk$^-$ and RAT-2tk$^-$ cells much more efficiently than supercoiled molecules. This finding implies that a DNA molecule with exposed ends is a better substrate for the recombination machinery of the cells. Consistent with this hypothesis are the studies with single-copy transformants which suggest that linear molecules are incorporated into the host chromosome through their ends. We have also observed that linear molecules can be recruited into concatemers by homologous recombination. The simplest interpretation of this observation is that many of the linear molecules must be recircularized before entry into the concatemer. Finally, if the concentration of linear molecules in the nucleus is high (~100 copies per cell), one even observes end-to-end ligation.

Therefore, it appears that in the nucleus competition exists for the ends of the donated DNA to be ligated into host DNA sequences, to themselves (resulting in recircularization), or to other exogenous DNA sequences (resulting in end-to-end ligation). Which event occurs is presumably controlled by the concentration of ends and the enzymatic machinery which mediates each reaction. The observation that linear molecules are a better substrate for transformation can be explained if linear molecules are an obligatory intermediate for incorporating exogenous DNA into the host chromosome. Consistent with this hypothesis is the observation that a high transformation efficiency can be obtained by using fewer than five supercoiled molecules when these molecules are coinjected with a few copies of any linear molecule containing homology to the supercoiled molecule (Capecchi, unpublished data). It will be of interest to determine whether the linear molecules in these transformants are found at the ends of the concatenate. A similar prediction of such a model is that the junction between the host chromosome and the head-to-tail concatemers generated by injecting linear molecules is at the ends of the linearized plasmid.

After injection of approximately 100 plasmid molecules per cell, we have isolated transformants in which as many as 50 to 100 plasmid molecules have been incorporated into the head-to-tail concatemer. This propensity for generating head-to-tail concatemers indicates that the cells have a very active enzymatic system for homologous recombination. It will be interesting to determine whether we can exploit this machinery to "target" a gene by homologous recombination to a specific chromosomal location. On the other hand, the very efficient joining of two DNA fragments in vivo irrespective of their sequence compositions may reflect the presence in somatic cells of a very active illegitimate or nonhomologous recombination system. The success of gene targeting experiments will clearly depend on the interplay of these two recombination systems. Pertinent to such experiments are the observations of Graessmann et al. (16) and Botchan et al. (8) that retransformation of revertant cells that still contain SV40 sequences leads to integration of SV40 at separate chromosomal sites and that integration of SV40 appears to involve homologous recombination, but only between a very limited number of nucleotide base pairs (34).

One model for generating head-to-tail concatemers with linear molecules which we have not addressed is the following: initially plasmid sequences could be recruited into a concatemer randomly by end-to-end ligation. However, due to the possible instability of long inverted repeats within the host genome, adjacent head-to-head or tail-to-tail plasmid sequences could be selectively removed, leading to the final head-to-tail concatemers. Such models are rendered unnecessary by our finding that concatemers which contain randomly oriented plasmid sequences (e.g., L356 and L357) are stable.

In theory, the homologous recombination ma-

chinery which forms head-to-tail concatemers could also be used to excise these concatemers. Thus, it was of interest to determine whether such structures are as unstable as their counterparts in bacteria (1). We have shown that for a number of transformants the sequences within the head-to-tail concatemers are stable even when the cells are grown under nonselective conditions. Varmus et al. (39) have reported excision of Moloney murine leukemia virus DNA from cellular DNA apparently by a homologous recombination event between the viral LTR sequences. The estimated frequency of this event was 10^{-6} to 10^{-7} per generation. Our method for estimating the instability of the head-to-tail concatemers would not have detected such a rare event. Head-to-tail duplications of SV40 and polyoma virus sequences in cultured mammalian cells appear to be less stable (11, 20, 22). A simple explanation of this difference would be that these sequences are excised by a mechanism which is dependent upon both host and viral factors (e.g., T-antigen in the excision of polyoma virus duplications [4]). On the other hand, eucaryotic organisms have probably evolved a mechanism for maintaining repeated sequences, such as ribosomal genes, even though such genes are clearly capable of undergoing amplification (37) and rearrangement (21). The stability of our head-to-tail concatemers may reflect such a mechanism.

In addition to the structure of the injected molecules, another parameter that strongly affects the transformation frequency is the presence or absence of enhancer sequences. The SV40 DNA sequence which enhances the DNA-mediated transformation frequency has been mapped to the 72-base pair (bp) repeat sequence located between nucleotides 107 and 250 of the SV40 late region (Luciw and Capecchi, unpublished data). In Fig. 1 we show that the SV40 *Hin*dIII-to-*Hpa*II fragment, which contains the SV40 origin of DNA replication, the 72-bp repeat, and the 21-bp repeat (located between the SV40 origin of DNA replication and the 72-bp repeat) contains the transformation enhancer activity. On the other hand, *Eco*RII fragment G, which contains the SV40 origin of DNA replication, the 21-bp repeat, and only part of one of the 72-bp repeat, shows no detectable enhancing activity. The SV40 *Nco*I fragment, which contains the 72-bp repeat and the 21-bp repeat but not the SV40 origin of DNA replication, exhibits full enhancer activity. Taken together, these results localize the transformation enhancer activity to a region surrounding the 72-bp repeat.

The same SV40 72-bp repeat has been shown to be required for transcription of SV40 T-antigen (6, 19) and to enhance transcription from a rabbit β-globin gene 100-fold (5). As in the DNA-mediated transformation assay, the amount of enhanced transcription is not critically dependent on the position of the 72-bp repeat within the rabbit β-globin recombinant plasmid. These observations invite speculation on the relationship between the enhancing effects of the same sequences on two different phenomena, DNA-mediated transformation and transient expression of certain genes in transfected cells.

Enhancer sequences may directly or indirectly stimulate recombination (for example, by making the recombinant plasmid a better substrate for recombination or by shuttling the recombinant plasmid to a cellular compartment with a high recombination activity). Alternatively, enhancer sequences may stimulate DNA-mediated transformation not by increasing the frequency of integration into the host chromosome, but by increasing the probability that a gene is active after its integration into the host chromosome. A gene with an associated enhancer sequence may be transcriptionally active in a normally silent chromosomal environment. In the latter model enhancer sequences may function by altering local chromatin structure so as to facilitate transcription of an associated gene. It is possible to distinguish between models in which the enhancer sequences affect integration and models that affect expression of the transforming gene after integration by measuring the incorporation of the exogenous sequences into the host chromosome in the absence of selection.

ACKNOWLEDGMENTS

We thank L. Fraser for expert technical assistance in all phases of this work and L. Vitto and V. Allen for expert technical assistance with in situ hybridization. We also thank P. Luciw for preparation of some of the DNA fragments used in the DNA fragment coinjection experiments.

LITERATURE CITED

1. **Anderson, R. P., and J. R. Roth.** 1977. Tandem genetic duplications in phage and bacteria. Annu. Rev. Microbiol. **31**:473–505.
2. **Anderson, W. F., L. Killos, L. Sanders-Haigh, P. J. Kretschmer, and E. G. Diacumakos.** 1980. Replication and expression of thymidine kinase and human globin genes microinjected into mouse fibroblasts. Proc. Natl. Acad. Sci. U.S.A. **77**:5399–5403.
3. **Bacchetti, S., and F. L. Graham.** 1977. Transfer of the gene for thymidine kinase-deficient human cells by purified herpes simplex viral DNA. Proc. Natl. Acad. Sci. U.S.A. **74**:1590–1594.
4. **Basilico, C., S. Gattoni, D. Zouzias, and G. Della Valle.** 1979. Loss of integrated viral DNA sequences in polyoma transformed cells is associated with an active viral A function. Cell **17**:645–659.
5. **Benerji, J., S. Rusconi, and W. Schaffner.** 1981. Expression of a β-globin gene is enhanced by remote SV40 DNA sequences. Cell **27**:299–308.
6. **Benoist, C., and P. Chambon.** 1981. *In vivo* sequence requirements of the SV40 early promoter region. Nature (London) **290**:304–310.

7. **Botchan, M., J. Stringer, T. Mitchison, and J. Sambrook.** 1980. Integration and excision of SV40 DNA from the chromosome of a transformed cell. Cell **20**:143–152.
8. **Botchan, M., W. Topp, and J. Sambrook.** 1978. Studies on simian virus 40 excision from cellular chromosomes. Cold Spring Harbor Symp. Quant. Biol. **43**:709–719.
9. **Capecchi, M. R.** 1980. High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. Cell **22**:479–488.
10. **Capecchi, M. R., R. A. Vonder Haar, N. E. Capecchi, and M. M. Sveda.** 1977. The isolation of a suppressible nonsense mutant in mammalian cells. Cell **12**:371–381.
11. **Chan, H. W., M. A. Israel, C. F. Garon, W. P. Rowe, and M. A. Martin.** 1979. Molecular cloning of polyoma virus DNA in *Escherichia coli*: lambda phage vector system. Science **203**:887–892.
12. **Chia, W., and P. W. J. Rigby.** 1981. Fate of viral DNA in nonpermissive cells infected with simian virus 40. Proc. Natl. Acad. Sci. U.S.A. **78**:6638–6642.
13. **Della Valle, G., R. G. Fenton, and C. Basilico.** 1981. Polyoma large T antigen regulates the integration of viral DNA sequences into the genome of transformed cells. Cell **23**:347–355.
14. **DeLorbe, W. J., P. A. Luciw, H. M. Goodman, H. E. Varmus, and J. M. Bishop.** 1980. Molecular cloning and characterization of avian sarcoma virus circular DNA molecules. J. Virol. **36**:50–61.
15. **de Saint Vincent, B. R., S. Delbrück, W. Eckhart, J. Meinkoth, L. Vitto, and G. Wahl.** 1981. The cloning and reintroduction into animal cells of a functional CAD gene, a dominant amplifiable genetic marker. Cell **27**:267–278.
16. **Graessmann, A., M. Graessmann, W. C. Topp, and M. Botchan.** 1979. Retransformation of a simian virus 40 revertant cell line which is resistant to viral and DNA infections by microinjection of viral DNA. J. Virol. **32**:989–994.
17. **Graessmann, M., and A. Graessmann.** 1976. "Early" simian-virus-40-specific RNA contains information for tumor antigen formation and chromatin replication. Proc. Natl. Acad. Sci. U.S.A. **73**:366–370.
18. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
19. **Gruss, P., R. Dhar, and G. Khoury.** 1981. Simian virus 40 tandem repeated sequences as an element of the early promoter. Proc. Natl. Acad. Sci. U.S.A. **78**:943–947.
20. **Israel, M. A., H. W. Chan, M. A. Martin, and W. P. Rowe.** 1979. Molecular cloning of polyoma virus DNA in *Escherichia coli*: oncogenicity testing in hamsters. Science **205**:1140–1142.
21. **Krystal, M., P. D'Eustachio, F. H. Ruddle, and N. Arnheim.** 1981. Human nucleolus organizers on nonhomologous chromosomes can share the same ribosomal gene variants. Proc. Natl. Acad. Sci. U.S.A. **78**:5744–5748.
22. **Lania, L., S. Boast, and M. Fried.** 1982. Excision of polyoma virus genomes from chromosomal DNA by homologous recombination. Nature (London) **295**:349–350.
23. **Maitland, N. J., and J. K. McDougall.** 1977. Biochemical transformation of mouse cells by fragments of herpes simplex virus DNA. Cell **11**:233–241.
24. **Maniatis, T., A. Jeffrey, and D. G. Kleid.** 1975. Nucleotide sequence of the rightward operator of phage λ. Proc. Natl. Acad. Sci. U.S.A. **72**:1184–1188.
25. **McKnight, S. L., E. R. Gavis, R. Kingsbury, and R. Axel.** 1981. Analysis of transcriptional regulatory signals of the HSV thymidine kinase gene: identification of an upstream control region. Cell **25**:385–398.
26. **Mougneau, E., F. Birg, M. Rassoulzadegan, and F. Cuzin.** 1980. Integration sites and sequence arrangement of SV40 DNA in a homogeneous series of transformed rat fibroblast lines. Cell **22**:917–927.
27. **Mulligan, R. C., and P. Berg.** 1980. Expression of a bacterial gene in mammalian cells. Science **209**:1422–1427.
28. **Perucho, M., D. Hanahan, and M. Wigler.** 1980. Genetic and physical linkage of exogenous sequences in transformed cells. Cell **22**:309–317.
29. **Rigby, P. W., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
30. **Robins, D. M., S. Ripley, A. S. Henderson, and R. Axel.** 1981. Transforming DNA integrates into the host chromosome. Cell **23**:29–39.
31. **Scangos, G., and F. H. Ruddle.** 1981. Mechanisms and applications of DNA-mediated gene transfer in mammalian cells—a review. Gene **14**:1–10.
32. **Smiley, J. R., D. A. Steege, D. K. Juricek, W. P. Summers, and F. H. Ruddle.** 1978. A herpes simplex virus 1 integration site in the mouse genome defined by somatic cell genetic analysis. Cell **15**:455–468.
33. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
34. **Stringer, J. R.** 1982. DNA sequence homology and chromosome deletion of a site of SV40 DNA integration. Nature (London) **296**:363–366.
35. **Swanstrom, R., W. J. DeLorbe, J. M. Bishop, and H. E. Varmus.** 1981. Nucleotide sequence of cloned unintegrated avian sarcoma virus DNA: viral DNA contains direct and inverted repeats similar to those in transposable elements. Proc. Natl. Acad. Sci. U.S.A. **78**:124–128.
36. **Szybalska, E. H., and W. Szybalski.** 1962. Genetics of human cell lines. IV. DNA-mediated heritable transformation of a biochemical trait. Proc. Natl. Acad. Sci. U.S.A. **48**:2026–2034.
37. **Tantravahi, U., R. V. Guntaka, B. F. Erlanger, and O. J. Miller.** 1981. Amplified ribosomal RNA genes in a rat hepatoma cell line are enriched in 5-methylcytosine. Proc. Natl. Acad. Sci. U.S.A. **78**:489–493.
38. **Topp, W. C.** 1981. Normal rat cell lines deficient in nuclear thymidine kinase. Virology **113**:408–411.
39. **Varmus, H. E., N. Quintrell, and S. Ortiz.** 1981. Retroviruses as mutagens. Insertion and excision of a nontransforming provirus alter expression of a resident transforming provirus. Cell **25**:23–36.
40. **Wahl, G. M., C. Vitto, R. A. Padgett, and G. R. Stark.** 1982. Amplified CAD genes in Syrian hamster chromosomes localized by a highly sensitive method for in situ hybridization. Mol. Cell. Biol. **2**:308–319.
41. **Wigler, M., S. Silverstein, L. S. Lee, A. Pellicer, Y. C. Cheng, and R. Axel.** 1977. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell **11**:223–232.
42. **Wigler, M., R. Sweet, G. K. Sim, B. Wold, A. Pellicer, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel.** 1979. Transformation of mammalian cells with genes from procaryotes and eucaryotes. Cell **16**:777–785.