Cotransfer of circular and linear prokaryotic and eukaryotic DNA sequences into mouse cells

(DNA transfer/bacterial plasmid/human globin gene/integration)

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ABSTRACT We have attempted to introduce some eukaryotic and prokaryotic DNA sequences into mouse fibroblasts. Purified herpes thymidine kinase gene (tk) was introduced into mouse cells. The presence of the herpes tk gene was established by gel electrophoresis, sensitivity to the purine analog acycloguanosine, and Southern blot hybridization. We utilized two different methods to introduce nonselectable markers into mouse cells. Bacterial plasmid pBR322 was ligated to herpes tk and used for transfection. All cells that were TK+ also contained the plasmid sequences. In the second method, pBR322 DNA was mixed with herpes tk DNA and presented to mouse cells. TK+ cells were tested for pBR322 sequences by blot hybridization. The frequency of unlinked cotransfer was greater than 40%. When the circular plasmid containing pBR322 and tk was used for transfection, each of the resulting transfectants acquired several copies of the plasmid. Most of the copies were associated with high molecular weight DNA in the cell. In addition, we found that some of the plasmid molecules may exist as free circular molecules. Using the nonligated cotransfer method, we introduced purified human β-globin sequences into the recipient cells. We were unable to detect any transcripts of the human β-globin gene at a level greater than or equal to 10 molecules per cell

Introduction of relatively large amounts of genetic material from one mammalian cell into another can be accomplished by somatic cell fusion (see ref. 1 for a review), microcell fusion (2), or chromosome-mediated transfer (3, 4). All of these methods are useful in the genetic analysis of mammalian genomes. The smallest segment of DNA transferred by any of these methods is approximately 40 kilobases (kb) long (5). The discovery that purified DNA from herpes simplex virus (HSV) can be used for infection (6) and to transfer the thymidine kinase gene (tk) (7, 8) provided the impetus for studies aimed at introducing DNA from various sources into mouse fibroblasts (9, 10, 11). Mantei et al. (12) have shown that specific nonselectable sequences can be efficiently transferred when they are ligated to the HSV tk sequence. It has been shown that nonselectable markers can be transferred at a high efficiency along with the HSV tk sequences even when they are not covalently linked to tk (10, 11).

We have studied the efficiencies of ligated and nonligated cotransfer of prokaryotic and eukaryotic circular or linear DNA sequences into mouse fibroblasts. We report here that bacterial plasmid pBR322 and human chromosomal β-globin genes can be transferred into mouse L cells by these methods. The results indicate that in all cases the donor DNA is associated with high molecular weight DNA in the recipient cell and that there is no site specificity for integration of these molecules.

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MATERIALS AND METHODS

Cells. Mouse L cells deficient in thymidine kinase (TK) were obtained from R. Axel (Columbia University) and F. Ruddle (Yale University). They were maintained in Dulbecco's modified Eagles medium (GIBCO) supplemented with 10% fetal calf serum (Flow) and penicillin and streptomycin.

DNA. The 5.1-kb Kpn I fragment containing tk was isolated from total HSV type 1 DNA by the method described by Wigler et al. (8). pHSV106, a plasmid carrying the 3.4-kb BamHI fragment of HSV 1 containing the tk sequences, was kindly provided by S. McKnight (Carnegie Institution). pβ1, a plasmid carrying a 4.4-kb Pst I subfragment of λ clone H3G1 (13) that contains human DNA having coding and noncoding sequences for β-globin, was generously provided by T. Maniatis (California Institute of Technology). The structures and some of the restriction enzyme sites of the various DNAs used in these experiments are shown in Fig. 1. Salmon sperm DNA was purchased from Sigma.

DNA Transfection. DNA transfection was achieved by the calcium phosphate precipitation technique described by Wigler et al. (8). Transfectants were selected by the hypoxanthine/aminopterin/thymidine (HAT) selection system (14). The amounts of HSV tk and other DNA used varied from experiment to experiment, but in all cases the final DNA concentration was made up to 20 μg/ml by adding appropriate amounts of salmon sperm DNA.

Acycloguanosine Tests. Acycloguanosine [9-(2-hydroxyethoxymethyl)guanine] was kindly provided by G. Elion (Wellcome Laboratories, Research Triangle Park, NC). Cells were treated with various concentrations of it and their rates of survival or colony-forming ability were determined by conventional methods.

Detection of DNA Sequences. Cells were expanded and DNA was isolated from them by a method described by Pellicer et al. (15). DNA was digested to completion with restriction enzymes EcoRI, BamHI, Kpn I, and Xba I obtained from Bethesda Research Laboratory. Digested DNA was electrophoresed in 0.8% horizontal agarose gels at 2 V/cm for 15–18 hr. The DNA was denatured with alkali and transferred to nitrocellulose filters (Millipore) by the method described by Southern (16). The filters were hybridized with nick-translation 32P-labeled DNA as indicated. The specific activity of the probe usually was 1–2 × 106 cpm/μg. The filters were washed and exposed to x-ray film (Kodak X-Omat) for 2–21 days. λ or adenovirus DNA digested with appropriate enzymes was used to provide size markers.

Abbreviations: kb, kilobase(s); tk, thymidine kinase gene; TK, thymidine kinase; HSV, herpes simplex virus; HAT, hypoxanthine/aminopterin/thymidine.
Fig. 1. Structure of DNA used for transfection. O, EcoRI cleavage site; Δ, BamHI cleavage site; ●, Pst I cleavage site. (A) pHSV106, bacterial plasmid pBR322 carrying the 3.4-kb BamHI fragment of HSV 1. Wavy line represents HSV sequences. (B) Human β-globin-containing DNA. Heavy line represents β-globin gene sequences. (C) Bacterial plasmid pBR322 carrying the 4.4-kb human DNA shown in B.

Experimental Design. DNA from different sources was mixed with or ligated to specific DNA fragments containing HSV 1 tk gene, and calcium phosphate precipitates of such DNA were presented to mouse L cells deficient in TK. HAT-resistant colonies arose from these experiments. Several of these colonies from each experiment were isolated and expanded for analysis. The sources of DNA in these experiments and the yield of HAT-resistant colonies are shown in Table 1.

RESULTS

Nature of tk in Transfectants. Mouse LMTK− cells were not known to revert. Thus, the HAT resistance of the transfected can be inferred to be due to the transfer of HSV tk. To confirm this, cell extracts were subjected to electrophoresis in polyacrylamide gels and slices of the gel were examined for tk activity. The electrophoretic mobility of the tk in transfecants corresponded to that of HSV tk (results not shown). These cell lines were also tested for resistance to the purine analog acycloguanosine. Representative results of this assay are shown in Fig. 2. Cells infected with or containing HSV tk were highly sensitive to acycloguanosine whereas mouse TK+ cells were resistant. All of the transfecants behaved like cells containing HSV tk.

Nonligated Transfer of pBR322 Sequences. Intact circular plasmids of pBR322 or molecules that were linearized by digestion with Pst I were mixed with the 5.1-kb Kpn I fragment and precipitated onto mouse cells. DNA from the resulting transfecant cell lines containing HSV tk was digested with EcoRI, denatured, and transferred to nitrocellulose filters. The filters were hybridized with nick-translated 32P-labeled pBR322 DNA. Representative results are shown in Fig. 3. Under the conditions of hybridization pBR322 DNA did not hybridize to mouse DNA. Several of the cell lines tested contained DNA homologous to pBR322. There were one to five bands in the positive lines. The sizes of the DNA, containing homologous

Table 1. Frequency of transfecants in different experiments

<table>
<thead>
<tr>
<th>Source of tk</th>
<th>Nonselected marker DNA</th>
<th>No. of exps.</th>
<th>Total colonies</th>
<th>Colonies/dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kpn fragment</td>
<td>None</td>
<td>10</td>
<td>553</td>
<td>11</td>
</tr>
<tr>
<td>Kpn fragment</td>
<td>pBR322</td>
<td>5</td>
<td>63</td>
<td>2.5</td>
</tr>
<tr>
<td>pHSV106</td>
<td>None</td>
<td>2</td>
<td>361</td>
<td>18</td>
</tr>
<tr>
<td>pHSV106 Human β-globin</td>
<td>1</td>
<td>166</td>
<td>33</td>
<td></td>
</tr>
</tbody>
</table>

Transfections with the Kpn fragments were done at 5–10 tk equivalents per cell. Experiments with pHSV106 were done at 10–15 equivalents per cell. Kpn fragment refers to a 5.1-kb fragment obtained after digestion of HSV 1 DNA with Kpn I.

Fig. 2. Sensitivity of parental and transfecant cell lines to acycloguanosine. O, mouse L cells; ●, mouse L cell containing HSV tk; Δ, mouse L cell transfected with 5.1-kb Kpn I fragment.

Fig. 3. Detection of pBR322 sequences in transfecants. DNA was isolated from transfecant cell lines and digested to completion with EcoRI; 10 μg of the digested DNA was separated on 0.8% horizontal agarose gels, denatured, and blotted onto nitrocellulose filters. The filters were hybridized with nick-translated 32P-labeled pBR322 DNA. Lanes: 1, pBR322 DNA digested with EcoRI; 2–6, cell lines cotransfected with pBR322 DNA (2, LGU9f; 3, BGU1b; 4, BGC1b; 5, BGC2b; 6, LGU1a; 7, BGC9a). Size marker denotes kb.
regions to pBR322, were different in each positive line. In several cases, the bands were larger than native linear pBR322. We have examined a total of 40 cell lines, and 12 of these lines containing the bacterial plasmid DNA did not differ significantly whether the pBR DNA was circular or linear. These results indicate that the pBR322 DNA is taken up by mouse fibroblasts and is associated with DNA of high molecular weight, presumably host DNA.

**Ligated Transfer of pBR322 Sequences.** pBR322 DNA was digested with *Bam*HI and ligated to a purified 3.4-kb fragment of HSV DNA which includes the *tk* gene (S. McKnight, personal communication). The resulting plasmid is shown in Fig. 1A. DNA from this circular plasmid (pHSV106) was mixed with human β-globin DNA (see below) and salmon sperm DNA, precipitated, and presented to LMTK<sup>-</sup> cells. HAT-resistant colonies were isolated and their DNA was analyzed as before; 10 different cell lines were examined extensively. These cell lines were used to examine the entry of pBR322 and human globin sequences. The DNA was digested with *Eco*RI or *Bam*HI and transferred to filters. The filters were hybridized with p51, which contains pBR322 and human globin sequences (Fig. 1C), or pHSV106.

The results from the latter experiment are shown in Fig. 4. Each of the cell lines contained multiple bands. *Eco*RI digests of all cell lines revealed three bands in common, of sizes 4.6, 2.2, and 1.0 kb. When digested with *Bam*HI all cell lines were found to contain 4.4- and 3.4-kb bands in common. The other bands were unique to each of the independently derived cell lines. Digestion of pHSV106 with *Eco*RI resulted in three fragments, 4.6, 2.2, and 1.0 kb. Of these, the 4.6- and 1.0-kb fragments contained pBR322 sequences and the 2.2-kb fragment contained exclusively HSV *tk* sequences. Similarly, digestion of pHSV106 with *Bam*HI resulted in 4.4- and 3.4-kb fragments corresponding to pBR322 and *tk* sequences, respectively. The common bands between different transfectants that we have observed can be explained by postulating that (i) each of the cell lines contains multiple copies of pHSV106 and some of them exist as free circular plasmids or (ii) multiple copies of pHSV106 are covalently associated with high molecular weight DNA and one of these molecules linearized by opening within the pBR322 sequence, leaving intact a 2.2- and a 1.0-kb *Eco*RI fragment and a 3.4-kb *Bam*HI fragment, and integration of another molecule led to an intact 4.6-kb *Eco*RI or 4.4-kb *Bam*HI fragment, or (iii) two or more molecules integrated tandemly in the cells. Some of these possibilities can be distinguished by digesting DNA with an enzyme that cleaves once within pHSV106 and analyzing the resulting DNA. If there is free pHSV106, this DNA should be detectable as a 7.8-kb band corresponding to the free plasmid.

**Kpn I Digestion of Transfectant Cell DNA.** DNA from the 10 transfectants was digested with *Kpn I*, an enzyme that has one cleavage site in pHSV106. The blots prepared from this DNA were hybridized with pHSV106. Each of the cell lines exhibited multiple bands (Fig. 5). Most of the bands were either smaller or larger than the native plasmid. A remarkable feature about all of these cell lines was the presence of a common band in the size range 7.7–8.0 kb. These results indicate that either the first or third of the hypotheses proposed above best explains the results.

**FIG. 4.** Detection of pHSV106 sequences in LGC series of transfectants. DNA was digested with *Bam*HI, separated by gel electrophoresis, transferred to filters, and hybridized with pHSV106 sequences. Note the 4.4- and 3.4-kb bands in all transfectants. Also note the slightly faster mobility of pHSV 106 DNA (lane 7) in the absence of high molecular weight DNA. Lanes: 1, p51 digested with *Eco*RI; 2, LGC21f; 3, LGC21e; 4, LGC21a; 5, LGC22b; 6, LGC20c; 7, pHSV106 digested with *Bam*HI.

**FIG. 5.** Nature of pHSV106 in transfectants. DNA was digested with *Kpn I* and blot/hybridized with labeled pHSV106 DNA. Lanes: 1, LGC21f; 2, LGC21a; 3, LGC21e; 4, LGC22a; 5, LGC22c; 6, LGC22d.
FIG. 6. Detection of human $\beta$-globin-specific sequences in transfectants. Two halves of a gel containing duplicate samples digested with BamHI were blot/hybridized with pBR322 (A) or p$\beta$1 (B). Note the 1.8-kb bands in lanes 3 and 4 in B which are globin specific. Lanes: 1, p$\beta$1(Bam); 2, LGC21F; 3, 21a; 4, 21e; 5, 20c; 6, 20b; 7, 22a; 8, 22c; 9, 22d.

Transfer of Human $\beta$-Globin Sequences. To study the differences between uptake and integration of prokaryotic and eukaryotic DNA sequences, we mixed pHS106 DNA with purified chromosomal human $\beta$-globin sequences (p$\beta$1 digested with Pst I) and salmon sperm DNA, precipitated the DNA, and presented it to LMTK$^-$ cells. HAT-resistant colonies were isolated and their DNA was tested by using pBR322 and p$\beta$1 DNAs as probes. The results are shown in Fig. 6. Samples were digested with BamHI and electrophoresed in duplicate lanes in two halves of a gel. The DNA from the two halves was transferred to separate nitrocellulose filters and hybridized with the two probes. The two probes share pBR322 sequences; in addition, p$\beta$1 contains human $\beta$-globin-specific sequences. Sequences detectable with p$\beta$1 and not detectable with pBR322 must be globin specific. Two of the eight cell lines shown contained a 1.8-kb fragment that hybridized to p$\beta$1. Digestion of p$\beta$1 with BamHI resulted in a globin-specific 1.8-kb fragment. We have tested a total of 10 cell lines, of which 7 are of clearly independent origin. Three of these cell lines contained globin specific sequences. Thus, the frequency of cotransfer of globin sequences in this experiment is greater than 40%.

Expression of Foreign DNA Sequences in L Cells. All of the transfectants are selected for expression of tk and we have shown that they all express HSV sequences. In order to determine if sequences whose expression is not necessary for cell survival are expressed, we tested the RNA from these transfectants for globin-specific sequences. Total RNA was isolated by phenol extraction at $60^\circ$C and hybridized to $^3$H-labeled human $\beta$-globin cDNA (specific activity, $10^7$ cpm/$\mu$g). None of the three cell lines that contain the $\beta$-globin gene coding sequences or any of the other seven cell lines tested contained $\beta$-globin mRNA. Our hybridization method is sensitive enough to detect 10 RNA molecules per cell.

DISCUSSION
We have studied cotransfer of prokaryotic and eukaryotic sequences into mouse fibroblasts along with sequences coding for HSV TK. The efficiency with which the tk gene can be transferred in these experiments is comparable to that reported by others (8, 10). There are several lines of evidence that indicate that the tk is of HSV origin. Transfection with LMTK$^-$ DNA or salmon sperm DNA does not result in HAT-resistant colonies (ref. 10; unpublished observations). The electrophoretic properties of the enzyme as well as its sensitivity to acycloguanosine prove its viral origin. The presence of intact 3.4-kb fragments in these transfected cell lines (Fig. 4) provide conclusive evidence for the physical presence of intact HSV tk gene sequences. The large difference in sensitivity of cells having mammalian tk compared to HSV tk makes this drug useful in rapidly screening large numbers of cell lines for HSV tk. This might prove especially useful in experiments involving transfer of viral tk when the recipient cells are either more resistant to
transfection or have a rate of reversion that is not much different from rates of transfection.

When circular molecules of pBR322 were mixed with linear molecules of HSV DNA and presented to mouse cells, 30% of the cell lines containing tk were also found to carry pBR322 sequences. The ratio of input tk gene equivalents to pBR322 sequences in these experiments varied from 1:2 to 1:20. Using a ratio of 1:1000 or greater, Wigler and coworkers were able to show up to 80% cotransfer of tk and pBR322 sequences. Among the cell lines that contained them, the number of pBR322 sequences was low, ranging from one to a maximum of five. The pBR322 sequences are associated with high molecular weight DNA, presumably of mouse origin. The size of DNA fragments that hybridized to pBR322 sequences varied from cell line to cell line. If the pBR322 sequences integrated into the mouse genome, they integrated at different sites as defined by restriction enzyme cleavage sites. We cannot rule out the possibility that the DNA is integrated into the carrier DNA which in turn may or may not be associated with cellular DNA.

The lack of specificity in the integration process is further demonstrated in our studies involving transfer of pBR322 and HSV tk ligated to each other. When circular molecules consisting of these two DNA sequences were used in transfections, all cell lines that contained tk also contained pBR322. These results are comparable to those of studies by Mantel et al. (12) in which they were able to transfer rabbit β-globin sequences into mouse cells by a similar method. Digestion of DNA from our transfectants revealed that all cell lines had some bands of common sizes in addition to bands of different sizes. The presence of bands of common sizes in all cell lines could be due to nonintegration, continuous production of circular molecules, or integration of multiple molecules in tandem or at multiple sites. If two plasmid molecules have integrated, each of them must have used a different site within it for integration.

Studies with an enzyme that cuts once within the plasmid provided information on this subject. The multiple bands we observed indicate that there are many DNA copies homologous to pHSV106. The common band (7.8 kb) seen in all of the transfectants indicates that there are either free circular or tandemly integrated forms of the plasmid. In all previous reports (8, 11, 15, 17) there was evidence for a single copy of tk gene in the transfectant cells. The results obtained in this study indicate that more than one copy of tk can be present in the recipient cells. Huttner and colleagues (11) have reported results which indicate that, in some instances, when the donor DNA is circular the recipient cells contain circular forms of DNA. Our results are consistent with these. The exact state of these molecules (supercoiled or relaxed circles) and the mechanism by which these molecules might be able to persist in the circular form is not known.

Cotransfer of nonselectable genes can be achieved by ligation of the nonselectable DNA to HSV tk or by simply mixing it with tk DNA. This observation extends to both prokaryotic and eukaryotic sequences. We were able to show that human β-globin sequences can be introduced into mouse cells by the nonligated cotransfer method. Similar results have been reported with rabbit β-globin sequences (10) and human β-globin sequences (11). The number of copies of globin-specific DNA sequences is limited (Fig. 6) and is probably one in each of our positive transfectants. The analysis we have conducted indicates that the β-globin sequence-positive cell lines contain a stretch of DNA starting from about 1 kb from the 5' end of the beginning of the coding region and running to the second intervening sequence. Thus, we cannot be certain whether the whole coding region of the gene is present in these cell lines. We were not able to detect any human β-globin-specific RNA in these cells. Wold et al. (18) have reported that, in cells cotransfected with rabbit β-globin DNA, some of the cell lines contain rabbit β-globin sequences and two of these express β-globin RNA sequences at two and five copies per cell. Both of these cell lines, however, contain multiple copies of rabbit β-globin sequences (up to 20). Mantel et al. (12) have also reported rabbit β-globin mRNA sequences in their transfectants. The number of DNA copies in their cell lines was also much larger than we observed in our transfectants. The lack of detectable human β-globin RNA molecules in the transfectants can be attributed to the low copy number of these sequences. On the other hand it is possible that our cell lines are expressing globin mRNA but at levels too low to be detected by our assay.

In summary, we have been able to introduce prokaryotic and eukaryotic sequences into mouse cells by DNA transfection methods. In the ligated transfer, multiple copies of tk are integrated and these are associated with high molecular weight DNA. There is no site specificity for integration of these molecules. Nonselected markers can be introduced without ligating them to tk sequences. Most of the sequences seem to be associated with high molecular weight DNA in the cell, but a relatively small proportion also exist as free circular molecules.

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