Volume 9 Number 22 1981 Nucleic Acids Research

Recovery of recombinant bacterial plasmids from E. coli transformed with DNA from microinjected mouse cells

Peter J.Kretschmer†, Anne H.Bowman†, Michael H.Huberman, Linda Sanders-Haigh, Lillian Killos and W.French Anderson*

Laboratory of Molecular Hematology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20205, USA

Received 9 July 1981

ABSTRACT

We have previously described the isolation of thymidine kinase positive (TK^+), human beta-globin gene-containing colonies following co-microinjection of mouse TK^- L cells with two recombinant pBR322 plasmids, one containing the TK gene of herpes simplex virus type I (plasmid pXl), and the second containing a human genomic DNA fragment within which is the human beta-globin gene (plasmid pRKl). DNA isolated from one such clone was used in bacterial transformation experiments with a selection for tetracycline-resistant colonies (that is, for cells containing pRKl). A total of forty-two tetracycline-resistant colonies were isolated, thirty of which contained circular pRKl molecules identical to those originally injected. The remaining twelve colonies contained unique plasmids that were grouped into five different classes of recombinant molecules. All five of these unique recombinant classes appear to contain a common deletion endpoint occurring at a specific region of the pBR322 segment of pRKl. Four of the unique recombinant classes appear to have arisen from the deletion of a segment of a pRKl trimer or dimer molecule, while the fifth class appears to have resulted from recombination between pRKl and pXl followed by a deletion event within this recombinant. It is uncertain whether these deletions are occurring within the eukaryotic cell or upon subsequent transformation of the bacterial cell. If the latter, then the passage of the plasmid DNA through the eukaryotic cell alters a specific site of the pBR322 DNA in such a way that deletions can occur at a high frequency in this region when the plasmid DNA is introduced back into a bacterial cell. Thus, we have established a prokaryote-eukaryote-prokaryote DNA transfer and recovery system which should be useful in studies on DNA replication and the regulation of gene expression in higher eukaryotes.

INTRODUCTION

Transfer of genetic material from bacterial cells to the eukaryote yeast cell and back again to bacterial cells is now a routine procedure (1-3). A number of recent methods have been developed for the introduction of defined DNA fragments into higher order eukaryotic cells. These have included transformation of mouse thymidine kinase negative (TK^-) L cells with a herpes simplex TK DNA fragment together with other DNA fragments or plasmids (4-6), use of SV40 vectors containing globin DNA into monkey cells (7, 8), and microinjection of recombinant plasmids containing eukaryotic genes into nuclei of

mouse tissue culture cells (9, 10). The aim of all these experiments was essentially two-fold. The first was to establish methods by which genetic material, manipulated and prepared in bacterial cells, could be transferred to and functionally expressed in eukaryotic cells for the analysis of gene expression. The second purpose was the use of information gained from such experiments for the eventual genetic therapy of human patients. Indeed, transformation of stem cells in a whole mouse by eukaryotic DNA has been reported (11).

We report here the establishment of a prokaryote-eukaryote-prokaryote DNA transfer and recovery system. Previously, we described the isolation of TK^+ clones following co-microinjection of mouse TK^- L cell nuclei with two recombinant pBR322 plasmids, one containing the TK gene of herpes simplex virus and the second containing a human genomic DNA fragment within which is the human beta-globin gene (9). The present report describes the recovery of normal and unique plasmid molecules following transformation of E. coli with the DNA of one such microinjected TK^+ L cell clone.

MATERIALS AND METHODS

Bacterial and Plasmid Strains and DNA Methodology

The bacterial strain used for transformation in this study was PK290 [a $C600r^-_km^-_krec\ A^+$ strain originally described by Meselson and Yuan (12)]. Plasmids pBR322 (13), pRK1 (14), and pX1 (15) have been described and are illustrated in Fig. 1. Methods for maintenance and growth of bacterial strains, chloramphenicol amplification and extraction of plasmid DNA have been described previously (16). Antibiotics used were tetracycline HCl (ICN Pharmaceuticals, Inc.) and ampicillin (Parke-Davis) at 10 and 20 µg/ml, respectively. Conditions for the ligation of DNA fragments using T4 DNA ligase were exactly as described previously (16). DNA was extracted from mouse L cells as described previously (9).

Bacterial Transformation Procedure

The bacterial transformation procedure is basically that of Dagert and Ehrlich (17) with the following modifications. Transformation-competent cells were used for transformation eighteen hours after preparation resulting in a transformation frequency of greater than 10^7 transformants/µg of pBR322 DNA, which was routinely three-fold that observed at zero time. For transformation with L cell DNA, 1 µg of DNA in 10 µl of TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) was added to 100 µl of eighteen hour competent cells in chilled glass test tubes. Following incubation on ice for ten minutes, the

cells were incubated at 37°C for five minutes, 2 ml of L broth were added, and incubation at 37°C in the original test tube was continued for one hour. Aliquots (0.3 ml) of the resulting transformation mix were plated on L-agar plates containing 10 µg/ml of tetracycline and the plates incubated at 37°C for sixteen to thirty hours.

Restriction Endonuclease and Southern Blot Analysis

Restriction endonucleases were purchased from Bethesda Research Laboratories, New England Biolabs and Boehringer-Mannheim Biochemicals. Reaction conditions for all enzymes (for single and double digestions) were 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, except for Kpn I which was used in the buffer recommended by the supplier. Purified TK DNA fragment was isolated following Bam HI digestion of pXl plasmid DNA, agarose gel electrophoresis, and extraction of the 3.5 kb TK fragment from the gel as described previously (16). For use as probe in Southern blot experiments, this DNA was further purified by an additional gel electrophoretic and DNA extraction step before nick-translation. The 4.4 kb Pst I human globin gene-containing fragment (HGG) was prepared free of pBR322 sequences by isolation of this fragment from Pst I digested λ bacteriophage HβGl DNA (14, 18). Nick-translation of the DNA fragments with [^{32}P]-labeled nucleotide triphosphates to yield radioactive probes and conditions for Southern blot analysis were as previously described (16).

RESULTS

Extraction of DNA from Microinjected Mouse L Cells

The plasmid pXl is a pBR322 clone containing the 3.5 kb Bam HI herpes simplex type I virus fragment which codes for thymidine kinase (15). The plasmid pRKl is a pBR322 clone containing the 4.4 kb Pst I fragment of human genomic DNA which codes for adult beta-globin (14). Bacteria containing the pXl plasmid are ampicillin-resistant (Ap^R) and tetracycline-sensitive (Tc^S), while cells containing pRKl are tetracycline-resistant (Tc^R) and ampicillin-sensitive (Ap^S). Restriction endonuclease maps of these plasmids with respect to Eco RI, Bam HI, Sal I, Pvu II, Pst I, and Bgl II are illustrated in Fig. 1.

Following the microinjection of nuclei of mouse TK^- L cells with a mixture of pXl and pRKl plasmids (9), a number of TK^+ colonies were isolated. These colonies were shown not only to contain herpes simplex and human globin gene sequences, but also to transcribe these genes to give viral TK enzyme and low levels of human globin mRNA molecules. DNA extracted from one of these

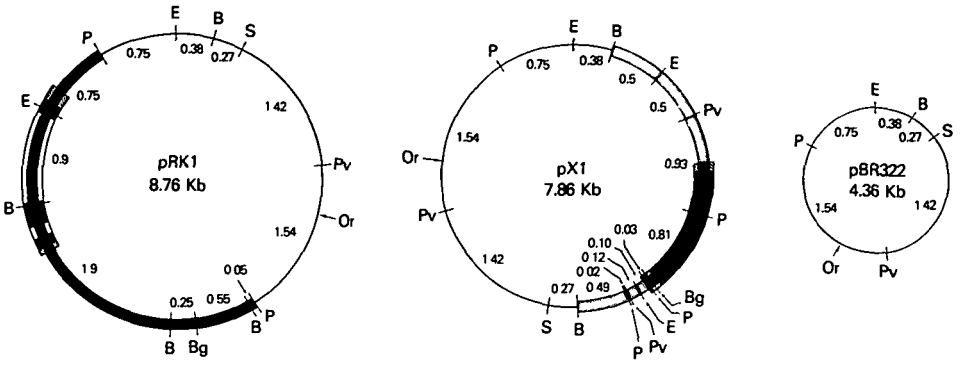


FIGURE 1.

Restriction Endonuclease Maps of pRK1, pX1 and pBR322.

Data for pRK1 were obtained in this laboratory; data for pBR322 were obtained in this laboratory and from the known pBR322 sequence (27); data for pX1 were obtained in part from Enquist et al. (15) and Wagner et al. (28). The restriction endonucleases used were: Eco RI (E), Bam HI (B), Sal I (S), Pvu II (Pv), Pst I (P), and Bgl II (Bg). The numbers are in kb. The thin portion of all circles represents pBR322. The thick solid portion of the pRK1 circle is the 4.4 kb Pst I fragment containing the human β globin gene. The globin gene itself is represented by the boxed area, from bottom to top: hatched, 5' nontranslated region; solid, 5' coding region; clear, first intron; solid, middle coding region; clear, second intron; solid, 3' coding region; hatched, 3' nontranslated region. The thick stippled portion of the pX1 circle is the 3.5 kb Bam HI fragment containing the herpes simplex type I thymidine kinase gene. The TK gene itself is represented by the boxed area, from bottom to top: hatched, 5' nontranslated region; solid, coding region; hatched, 3' nontranslated region. Or stands for origin of replication.

colonies, called C2B, was the focus of the experiments described in this report.

The C2B colony was expanded for sixty generations and DNA was extracted with care being taken to avoid contamination of the L cell DNA with extraneous plasmid DNA. As a control for plasmid contamination during the extraction procedure, DNA was simultaneously extracted from non-injected L cells and used in transformation experiments along with the DNA extracted from injected cells (see below and Table 1).

Transformation of Bacterial Cells with Mouse L Cell DNA

E. coli C600 derivatives deficient for the K restriction phenotype (r^-_k) in our culture collection were tested for optimum transformation frequency using essentially the method of Dagert and Ehrlich (17). Strain PK290 was consistently transformed with 0.05 ng of pBR322 at a frequency greater than

Table 1. Summary of Transformation Experiments and Plasmids Recovered

DNA Preparation					Plasmid Recovered		
DNA Source	Kpn I cut	Religation	µg Transformed	Number of Tc^R Colonies[a]	pRK1	Number of Recombinants	Example of Recombinants[b]
C2B	-	-	9	7 (-)	6	1	pPK682 (1)
C2B	+	+	11	13 (-)	9	4	pPK193 (4)
C2B	+	-	8	22 (4)	15	7	pPK578 (2)
							pPK579 (4)
							pPK576 (1)
Normal Mouse	-	-	41	0			

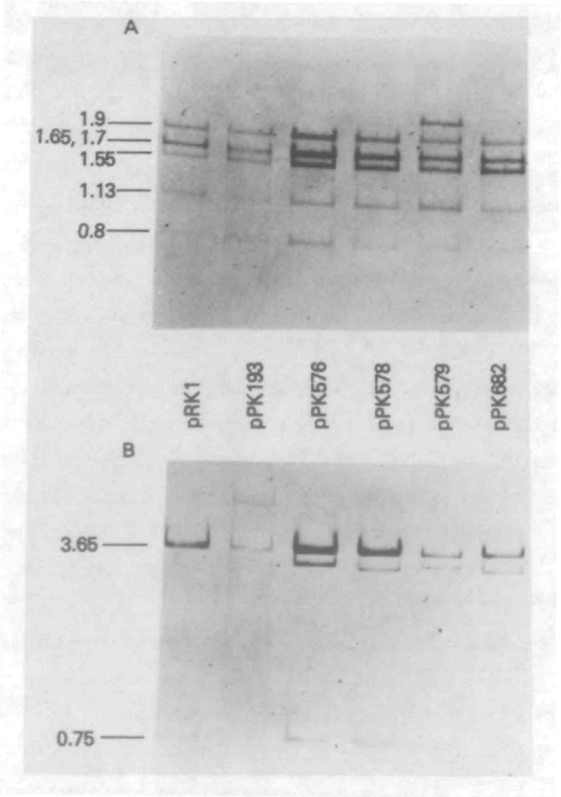
[a]Numbers in parentheses indicate number of Tc^R colonies that were also Ap^R.

[b]Numbers in parentheses indicate the total number of plasmids recovered for that particular class of unique recombinant plasmid.

10^7 transformants per µg of DNA, and as such was the strain chosen for the remainder of these studies. Extracted C2B DNA was used to transform E. coli in a number of ways. First, transformation without further manipulation. Second, in order to recover those pRK1 sequences which might be integrated linearly into mouse cellular DNA, the C2B DNA was digested with Kpn I followed by dilution and religation of this DNA. In restricting with Kpn I, which does not cut pRK1, any integrated pRK1 molecules should be excised as linear fragments bounded by, and contiguous with, mouse genomic DNA segments with Kpn I cohesive ends. The ligation of such a structure would result in a circular molecule that would be transformed at a much higher efficiency than linear molecules (P. Kretschmer and E. Schmader, unpublished results). Cutting and religation of C2B DNA was monitored by agarose gel electrophoresis and visualization of molecules using the electron microscope. From the latter, we estimated approximately 50% conversion of Kpn I cut linear to circular molecules. Finally, DNA that was Kpn I cut but not religated was also used in transformation experiments.

The results of these transformation experiments are summarized in Table 1. Nine µg of uncut C2B DNA yielded seven Tc^R colonies, giving a frequency of approximately one colony per µg of DNA. Eleven µg of C2B DNA that was Kpn I cut and religated yielded thirteen Tc^R colonies, again giving a frequency of about one transformant per µg of mouse DNA. Eight µg of C2B DNA that had been Kpn I cut but not religated yielded twenty-two Tc^R colonies, a frequency of approximately three transformants per µg of DNA. In contrast, 41 µg of normal mouse DNA that had been derived from non-microinjected TK^- L cells and transformed concurrently with the above experiments yielded no Tc^R transformants.

All forty-two Tc^R colonies were shown to contain plasmid molecules by routine plasmid DNA extraction procedures. In order to examine the recovered plasmids by restriction endonuclease and Southern blot analysis, we first derived accurate restriction maps for the two plasmids, pRK1 and pX1; the results are illustrated in Fig. 1. After consideration of the pRK1 restriction map, plasmid DNA preparations from the forty-two Tc^R colonies were initially screened using two sets of plasmid digestions in order to compare them with authentic circular pRK1. The first digestion was a Bam HI/Pst I/ Pvu II triple digestion (Fig. 2A), and the second was an Eco RI/Pst I double digestion (Fig. 2B). Using these criteria, six classes of plasmids were iden-



FIGUR E 2.

Initial Screening of Plasmids Recovered from Microinjected Mouse L Cell DNA.

In an initial screening process, plasmids were digested with Bam HI/Pst I/Pvu II (panel A) and with Eco RI/Pst I (panel B) and analyzed on a 1% agarose gel.

tified. Of the forty-two plasmid DNA preparations, thirty gave digestion patterns identical to that of pRK1. The remaining five classes of plasmids, exemplified by the pPK plasmids 193, 576, 578, 579 and 682 were clearly different from pRK1 and from each other (Fig. 2), and from here on are referred to as unique recombinant plasmids. In all, three additional plasmid preparations were found identical to pPK193, three additional plasmid preparations were found identical to pPK579, one additional plasmid preparation was found identical to pPK578, while pPK682 and pPK576 were single isolates (see also Table 1). It was subsequently found that bacterial cells containing the pPK579 recombinant molecule were Tc^R and Ap^R, while cells containing the remaining plasmids were Tc^R only.

Characterization of Unique Recombinant Plasmids

All five classes of recombinants were analyzed in a similar manner. The first step in the analysis was to obtain accurate restriction endonuclease maps of each class for comparison with the restriction maps of the microinjected plasmids, pRK1 and pX1. The restriction maps were derived following single and all possible double digestions of the plasmids with Eco RI, Bam HI, Pst I and Pvu II. In addition, individual restriction fragments were isolated and digested with appropriate restriction enzymes to confirm or clarify plasmid maps. The results of these experiments produced an unambiguous derivation of the restriction map for each recombinant class. Derivation of the restriction map of pPK579 is described below; data for the remaining unique recombinant plasmid classes are available on request.

The unique recombinant plasmid restriction maps so derived predicted that various segments would contain homology to either pBR322, the 3.5 kb Bam HI TK fragment, the 4.4 kb Pst I human globin gene-containing fragment (HGG) or to combinations of these fragments. Therefore, in the second analysis step, either the whole plasmid or appropriate restriction fragments from each were isolated, digested with appropriate enzymes, and the digests so generated subjected to Southern blot analysis with the appropriate nick-translated probes. Results from these experiments are shown and discussed below for plasmid class pPK579. The results of similar experiments with the remaining four classes are not shown (data available upon request).

Plasmid Class Represented by pPK579

The restriction endonuclease map of pPK579 is shown in Fig. 3. This map was derived from single and all possible double digestions involving Eco RI, Bam HI, Pst I and Pvu II. The fragments resulting from these digestions are listed in Table 2. The molecular weight of the plasmid is calculated to be

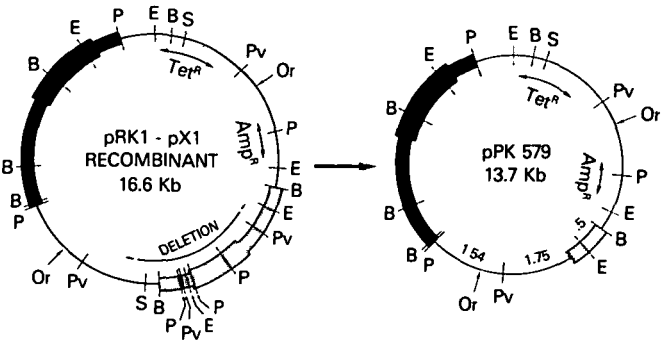


FIGURE 3.

Suggested Origin (from a pRK1-pX1 recombinant) and Restriction Endonuclease Map of Recombinant Plasmid pPK579. The precursor is represented as circle only for ease of conceptualization (see text).

For symbols, see legend to Fig. 1.

13.7 kb. pPK579 appears to be a pRK1/pX1 chimera with a 2.9 kb deletion which includes a considerable portion of the herpes simplex sequences as well as some pBR322 material (Fig. 3).

Upon examination of Pvu II and Pst I digestions, there appears to be a complete single copy of pRK1 present in the plasmid pPK579. Isolation and restriction endonuclease analysis of the 8.75 kb Pvu II fragment and the 4.4

Table 2. pPK579 Restriction Fragments

Eco RI	Eco RI Bam HI	Bam HI	Bam HI Pst I	Pst I	Eco RI Pst I	Eco RI Pvu II	Pvu II	Pst I Pvu II	Bam HI Pvu II
6.95*	4.0	4.35	3.8*	4.95*	3.65	5.2	8.75	4.4	2.8
4.35	3.35*	3.85*	3.25	4.4	3.6	2.3	4.95*	3.4*	2.7
1.5	1.9	2.8	1.9	4.35	3.3*	2.05		2.8	2.25*
0.88	1.5	1.9	1.65		0.88	1.75*		1.55	1.9
	0.9	0.8	1.15		0.75	1.5		1.55	1.7
	0.8		1.15		0.75	0.88			1.6
	0.5		0.8		0.75				0.8
	0.38		(0.05)						
	0.38								
13.68	13.71	13.7	13.75	13.7	13.68	13.68	13.7	13.7	13.75

*Indicates restriction fragment containing deletion endpoints.

and 4.35 kb fragments from a Pst I digest confirmed this impression. The remainder of the pPK579 DNA is thus represented by the 4.95 kb Pst I fragment and the 4.95 kb Pvu II fragment. Subsequent isolation of these two fragments and restriction endonuclease digestion revealed a structure compatible with that shown in Fig. 3.

Final proof of the restriction endonuclease map of pPK579 was obtained by isolation of the Eco RI fragments of pPK579 and subsequent digestion by various enzymes, some of which were subjected to Southern blot analysis as follows. Since the 4.4 kb human beta-globin gene fragment of pRK1 is intact in pPK579 (evidence from Pst I, Bam HI, Bam HI/Pst I and Eco RI digests), the remainder of pRK1 can be divided into three segments, the 0.75 kb Eco RI/Pst I segment, the 2.07 kb Eco RI/Pvu II segment, and the remaining 1.55 kb Pst I/ Pvu II segment. We then schematically recombined these three areas with the equivalent areas of homology on pX1 and obtained two different types of potential recombinants, one of which we are postulating is the precursor of pPK579. The first type of putative full recombinant between pRK1 and pX1 which would be derived by a cross-over event between the homologous 0.75 kb Pst I/Eco RI regions, would yield a plasmid molecule in which the human β globin gene fragment is only 1.1 kb away from the TK fragment. The second type of full recombinant has the homologous cross-over regions in either the 2.05 or the 1.55 kb segments mentioned above, and on this recombinant the human β globin gene and TK fragments would be on opposite sides of the molecule separated by at least 3 kb of pBR322 DNA. One can distinguish which full recombinant gave the deletion plasmid since in the first case (from the 0.75 kb Eco RI/Pst I segment) one should see a Pvu II fragment of 4.38 kb in size which spans the Tc and Ap regions of pBR322, leaving a 9.1 kb Pvu II fragment, whereas in the second type of full recombinant, one should see an 8.75 kb Pvu II fragment which is a complete copy of pRK1, thus leaving a 4.95 kb fragment. The latter is seen, and this therefore indicates that the second type of full recombinant is the one which gave rise to pPK579.

The homology regions indicated in Fig. 3 were derived in the following manner. Plasmid pPK579 and various isolated restriction fragments from it were subjected to restriction endonuclease and Southern blot analysis using [^{32}P]-labeled pBR322 and HGG as probes, in addition to the [^{32}P]-labeled 3.5 kb Bam HI TK fragment (hereafter called TK probe). When the pPK579 plasmid was digested with Eco RI/Pvu II and Eco RI/Bam HI, subsequent Southern blot analyses with the above three probes were totally consistent with the homology regions indicated in Fig. 3 (unpublished data, except that for Eco RI/Pvu II

digestion with TK as probe, which is shown in Fig. 4, tracks 5 and 6). When the isolated 6.95 kb Eco RI fragment of pPK579 was digested with Pvu II, only the 1.75 kb Eco RI/Pvu II fragment was shown to contain pBR322 and TK homology (Fig. 4, tracks 1-4), while the 5.2 kb fragment contained pBR322 homology (Fig. 4, track 2) and HGG homology (data not shown). Finally, the isolated 0.88 kb Eco RI fragment, when digested with Bam HI, revealed a 0.5 kb fragment containing only TK homology (Fig. 4, tracks 7 and 8), and a 0.38 kb fragment containing only pBR322 homology (data not shown).

Thus, we conclude that the Ap^R Tc^R plasmid pPK579 has arisen by deletion of a segment of DNA from a pRK1/pX1 chimeric molecule as illustrated in Fig.

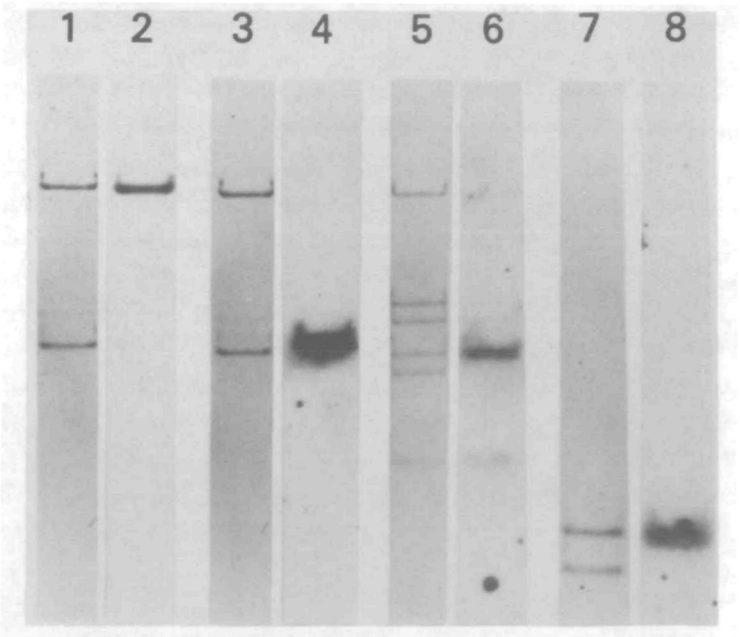


FIGURE 4.

Restriction Endonuclease and Southern Blot Analysis of pPK579.

The left track in each pair demonstrates the bands visualized by ethidium bromide staining, while the right track was obtained by transferring the DNA to nitrocellulose filters, annealing with [^{32}P]-labeled DNA as probe, followed by radioautography as described previously (16). Tracks 1 and 3 contain the 6.95 kb Eco RI fragment of pPK579 digested with Pvu II; track 5 contains pPK579 digested with Eco RI/Pvu II; and track 7 contains the 0.88 kb Eco RI fragment of pPK579 digested with Bam HI. The Southern blot analyses with these gels were with pBR322 probe for track 2, and TK probe for tracks 4, 6, and 8.

3. For ease of conceptualization, the precursor is denoted as a circle. It is not yet known, however, whether the precursor is present as an open or closed circle, or as part of a linear concatamer.

Plasmid Classes Represented by the pPK Plasmids 578, 682, 576 and 193

The remaining four plasmid classes were analyzed in a similar manner to that described above for pPK579.

The restriction map of pPK578 is shown in Fig. 5. As indicated in this figure, the data are consistent with pPK578 having been derived from a pRK1 trimer molecule by deletion of a continuous segment of DNA consisting of a majority of one HGG sequence and part of a pBR322 sequence. In an attempt to gain more information as to the length of HGG sequence homology remaining following the postulated deletion of DNA to form pPK578, the 3.1 kb Pvu II fragment encompassing the end points of this deletion was isolated and digested with Pst I and with Bam HI/Pst I, electrophoresed, and the resulting restriction endonuclease bands subjected to Southern blot analysis using HGG fragment as probe. The Pst I digest resulted in two 1.55 kb fragments and the Bam HI/Pst I digest resulted in 1.55, 1.5 and 0.05 kb fragments. Thus, any observed loss of hybridization intensity in the Bam HI/Pst I digest as com-

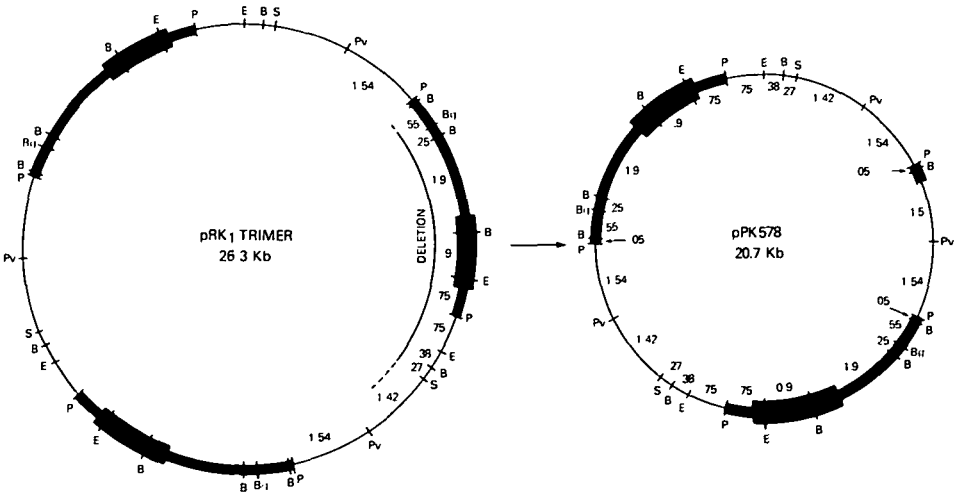


FIGURE 5.

Suggested Origin (from a pRK1 trimer) and Restriction Endonuclease Map of Recombinant Plasmid pPK578. The precursor is represented as a circle only for ease of conceptualization (see text).

For symbols, see legend to Fig. 1.

pared to the Pst I digest would be attributed to the loss of the 50 bp of HGG homology. Only a slight intensity difference was observed (despite equal amounts of DNA in each track; results not shown), indicating that a substantial length (perhaps 150-300 bp) of HGG sequence homology remains in the 1.5 kb Bam HI/Pvu II fragment, as indicated on the map of pPK578 in Fig. 5. However, the total HGG homology remaining is less than 550 bp, since the Bgl II site present in the HGG sequence (see pRK1 map of Fig. 1) is absent (unpublished results).

Restriction endonuclease maps of pPK682, pPK576, and pPK193 are illustrated in Fig. 6. As illustrated in this figure, these three plasmid classes can be postulated as being derived from three independent deletion events of a pRK1 dimer molecule. Since the Ava I site, located approximately 600 bp counterclockwise from the Pvu II site, is preserved in all these plasmids (data not shown), the deletion origin would appear to be within a 600 bp region between Ava I and Sal I. In fact, plasmids pPK578 and pPK579 also have a deletion endpoint in this region. Similar unique recombinant plasmids, arising from pX1 and isolated on ampicillin plates, were found to have a deletion origin in the same area (J. Curcio, unpublished data). Thus, it would appear that this region of the pBR322 molecule has become highly susceptible to deletion, but that the remaining endpoint in each case can be at a random position (see below and Discussion).

Are the Unique Recombinant Plasmids Formed in the Mouse Cell or the Bacterium?

We have made an effort to determine whether the recombination and deletion events giving rise to unique recombinant plasmids occur within the mouse cells, or whether they occur following transformation of the bacterial cells by the microinjected mouse cell DNA. First, we investigated the feasibility that the pRK1/pX1 recombinant, pPK579, could have been formed following transformation of a bacterial cell by separate circular pRK1 and pX1 molecules from the mouse cells. Ten µl of a DNA mixture containing 0.1 µg of pX1, and ten-fold dilutions of this mixture, were used to transform PK290 cells. The frequency of bacterial cells co-transformed by both plasmids ($Ap^R Tc^R$) was compared to the frequency of single transformation events by each plasmid (Ap^R or Tc^R). The results are shown in Table 3. No co-transformants were found below the dilution at which single transformation frequencies of 10^3 transformants/tube were observed. However, in the DNA recovery experiments described above, only one to three single transformation events per tube were obtained (Table 1). Therefore, in the recovery experiments, the possibility for co-transformation of bacterial cells by circular plasmids is virtually nonexistent.

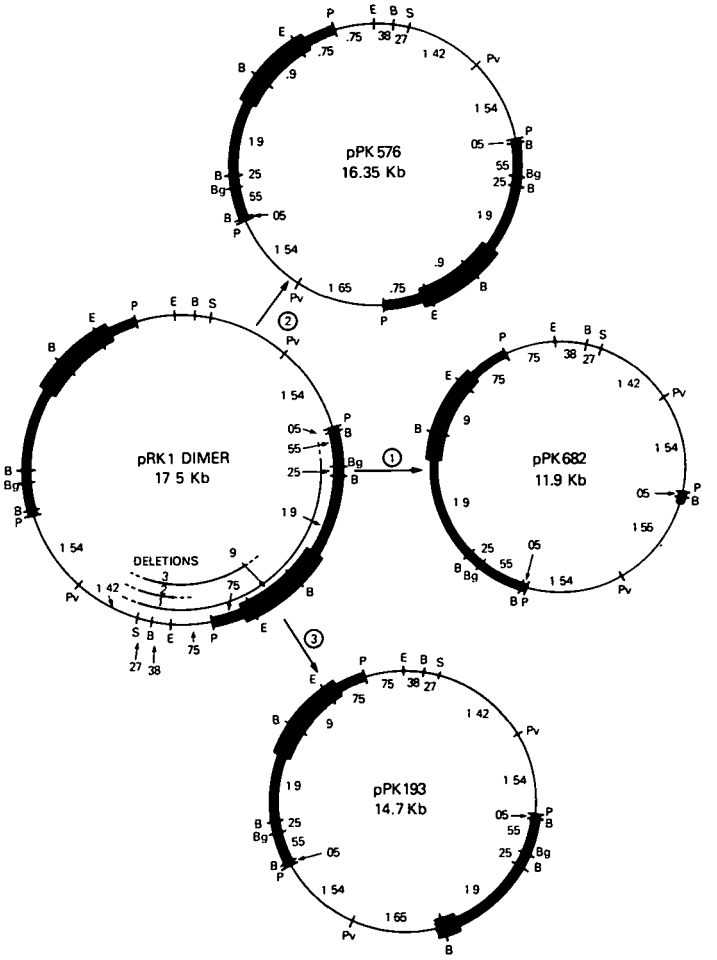


FIGURE 6.

Suggested Origin (from a pRK1 dimer) and Restriction Endonuclease Maps of Recombinant Plasmids pPK682, pPK576, and pPK193. The precursor is represented as a circle only for ease of conceptualization (see text).

For symbols, see legend to Fig. 1.

Do deletions occur, producing unique recombinant plasmids, when supercoiled pRK1 is used to transform C600 E. coli cells directly? Plasmid DNA was isolated from twenty Tc^R transformants obtained from the experiments of Table 3, line four (10^{-4} µg of input DNA). All twenty plasmid DNA preparations from these colonies were indistinguishable from that of pRK1 DNA as analyzed by

Table 3. Co-transformation of Bacterial Cells by pRK1 and pX1 Plasmid Molecules[a]

DNA (μg)[b]	Tc^R	Ap^R	Tc^R Ap^R
0.1	1.3×10^6	1.0×10^6	2.7×10^4
0.01	1.8×10^5	1.0×10^5	1.7×10^3
0.001	2.6×10^4	2.6×10^4	2.6×10^1
0.0001	1.2×10^3	1.0×10^3	0

[a]Total number of single transformation events for pRK1 (Tc^R) and pX1 (Ap^R), and of cotransformants of pRK1 and pX1 (Tc^R) and Ap^R).

[b]Number represents μg of each of pRK1 and pX1 added to one transformation tube containing 0.1 ml of competent cells (see Materials and Methods).

restriction endonuclease analyses (data not shown). It should be noted that greater than 95% of the pRK1 plasmid DNA used in the transformation experiments of Table 3 was in the dimer or higher forms (data not shown). Thus, a deletion event similar to those observed for the pPK plasmids (see above) could have occurred in the transformation experiments of Table 3 and still have resulted in tetracycline-resistant transformant colonies as were observed in the experiments of Table 1. The fact that we did not observe such deletions in this control experiment following transformation into bacterial cells is evidence that the deletion events observed in the experiments of Table 1 either occurred in the mouse cell or that the plasmid DNA was altered when it passed through the mouse cell in such a way that deletions can occur subsequent to transformation in the bacterial cell.

Was there a possibility that the deletion events observed in the experiments of Table 1 occurred following the transformation of linear (monomer-size) plasmid molecules into bacterial cells? Plasmid pRK1 DNA was linearized with Bgl II (see Fig. 1) and the linear DNA was isolated following agarose gel electrophoresis. This DNA was digested a second time with Bgl II and again linear pRK1 DNA was isolated from an agarose gel. Linear pRK1 DNA prepared in this manner was used to transform bacterial cells. Tc^R transformants were obtained at a frequency 1,000-fold less than that normally observed for pRK1. Plasmid DNA isolated from twelve different colonies was found to be identical to pRK1 on subsequent restriction analyses, including the presence of a Bgl II site. Although these data suggest that the observed deletion events might have occurred in the mouse cells, we could not rule out the possibility that all the Tc^R colonies were due to either transformation of the E. coli by linear concatamers followed by formation of Tc^R plasmids in the bacteria, or an occasional contaminating circular pRK1 molecule in the linear preparation.

In order to investigate the latter possibility, we designed the following experiment in which transformants resulting from such contaminating circular molecules could be distinguished from transformants resulting from linear molecules. The plasmid pRK1 was digested with Pst I and Bgl II, and the resulting 4.4 kb fragment (that is, pBR322 linearized at its Pst I site) was isolated and used to transform cells for tetracycline resistance. Tc^R colonies were isolated at a frequency 1,000-fold less than for that obtained by transformation of circular molecules (data not shown). The DNA preparations from ten such Tc^R colonies were examined using restriction endonuclease analysis. Preparations from Tc^R colonies due to transformation by uncut pRK1 molecules would, therefore, be expected to contain easily recognizable human β-globin sequences. In fact, all ten plasmid preparations were identical to pBR322, and all contained a Pst I site. That is, the Tc^R colonies resulted from transformation and subsequent circularization of linear pBR322 molecules.

The above experiments support the following two conclusions. First, the linkage of pRK1 with pX1 DNA appears to have occurred in the mouse cell since the likelihood of cotransformation of two separate linear or circular plasmid molecules into one bacterium is highly unlikely under the conditions used here. And second, the deletions producing the unique recombinant plasmids probably occurred by way of concatamers of linked pRK1 and pX1 molecules and that the deletion events and reassembly of circular plasmids then take place in the bacterial cells (see Discussion).

DISCUSSION

This study describes the successful recovery from transformed *E. coli* of a bacterial recombinant DNA molecule containing a human DNA insert following its microinjection, replication, and expression within mouse L cells. Despite the high efficiency of bacterial transformation systems used in this study, we feel that the plasmid DNA recovered (Table 1) was originally contained within the mouse cells, perhaps in the form of concatamers (see below), and were not due to contamination by extraneous plasmid DNA. As detailed in Table 1, while transformation of 28 μg of DNA extracted from microinjected cells resulted in 42 transformant colonies, no transformants were isolated from experiments in which 41 μg of DNA extracted in parallel from non-microinjected mouse L cells were used. However, in one experiment, we did isolate a contaminant plasmid that was currently in use within the laboratory (results not shown). Although this experiment was discarded, it serves to illustrate the dangers involved in experiments of such sensitivity.

From the frequency of plasmids recovered, it is clear that the recombinant bacterial plasmid DNA is being replicated within the mouse cell. At this stage, we cannot determine whether the majority of these sequences are integrated or non-integrated. It is interesting that none of the rescued plasmid DNA, including that obtained from cells transformed with Kpn-cut and religated DNA, contain mouse chromosomal DNA. This does not preclude the possibility of integration of the microinjected DNA. It is possible that mouse DNA-containing plasmids which may result from such treatment would be at a selective disadvantage in the bacterial transformation experiments of Table 1, either because they are too large compared to extrachromosomal plasmid DNA, or because such plasmids contain inserts of cellular DNA which reduce their viability.

Hanahan et al. (20) report the rescue of pBR322-SV40 recombinants following fusion of convected L cells with simian cells. These rescued plasmids were subsequently used to transform E. coli X1776 to ampicillin-resistance. In most instances, the cloned plasmids isolated from E. coli appeared to differ in their restriction pattern from the originally convected DNA. They also note that their transformation efficiencies were at least 100-fold lower than what would be predicted based on the amount of rescued plasmid DNA used in the transformation experiments. This anomaly has been recently explained (21) by a 'poison' sequence between pBR322 origin of replication and the Pvu II site, which, upon passage through simian cells, causes them to both replicate poorly and to transform E. coli with poor efficiency. In no instance have multimeric structures been found, although partial tandem duplications of the SV40 early region have been described (21). These results contrast with the data described in this paper, where 30/42 plasmids derived from the microinjected L cells were intact pRK1 molecules, and the remainder were multimeric with partial deletions.

Both our laboratory (9) and those of Huttner et al. (22) and Hsiung et al. (23) suggested that circular plasmid molecules might be present in mouse L cells that had been transformed with uncut recombinant bacterial plasmids based on evidence from Southern blot analyses. Direct evidence for this conclusion was not presented. Perucho et al. (24) have demonstrated that, upon transfer of DNA into mouse cells via the calcium-phosphate precipitation method, the host cells ligate the incorporated DNA into large concatameric structures, sometimes as large as 2,000 kb in size. These authors demonstrated recombination between the DNA used to transform cell lines and the carrier DNA used, either salmon sperm or hamster DNA. How-

ever, because of the large excess (20-fold) of carrier DNA used in these experiments and also the use of the calcium phosphate precipitate method of transformation, it is difficult to ascertain what would occur under more physiological conditions. By avoiding both carrier DNA and a calcium phosphate precipitate, we believe the microinjection method of introducing exogenous DNA to eukaryotic nuclei may afford a more physiologic approximation.

In order to determine directly whether the transforming activity of the microinjected L cell DNA is present in large molecular weight material (implying integration into mouse chromosomes or very large concatamers) or in smaller molecular weight DNA (circular plasmids or concatamers under approximately 100 kb in size), the fractionation procedure of Hirt can be used (25). Studies on another microinjected cell line, C_1, indicate that transforming activity is present in both the high and low molecular weight fractions of a Hirt extraction, with greater activity in the latter (Huberman and Anderson, unpublished results). The DNA of the Hirt supernatant was further fractionated by cesium chloride-ethidium bromide equilibrium sedimentation. Most of the transforming activity was found in the form II, III (relaxed) and not in the form I (supercoil) band. A number of unique recombinant plasmids, including an intact pRK1-pX1 chimera, have been isolated from these transformants. Thus, the data from this paper, as well as the complementary studies on cell line C_1, suggest, but do not prove, that at least some of the microinjected plasmid DNA is present extrachromosomally as tandem repeats. It is possible that this low molecular weight material arose from integrated material which had been sheared and remained in the Hirt supernatant.

The existence and frequency (4/42 colonies) of the unique recombinant plasmid pPK579 indicates that, regardless of the way in which the microinjected plasmid DNA is replicated, the linking of pRK1 and pX1 within mouse L cells is not totally happenstance. It should be emphasized that every plasmid recovered was mapped so that 4/42 is a true frequency. There is no obvious reason why these particular plasmids (the five unique classes) should offer any selective advantage to the bacteria transformed. Thus, these recombination/deletion events do not appear to be random rearrangements as are often seen in DNA transfer experiments (26). Rather, the experiments reported here suggest that a region in the commonly used plasmid vector, pBR 322, has become highly susceptible to deletion events as a result of its presence in the mouse cell. This region, perhaps only a single site, occurs near the end of the Tc^R gene complex. The fact that circular or linearized pRK1 (or pBR322) molecules do not undergo deletion upon transformation of bacteria implies that, if the

deletion events are occurring in the bacterial cell, then the DNA in the region of the deletion site(s) must be altered in the mouse cell so that this site is recognized in the bacterium as a deletion point. Perhaps microinjected DNA is acted on by an enzyme(s) in the mouse L cell to produce a different pattern of methylation (or other alteration which does not affect the base sequence). Such site-specific DNA-modifying enzymes, if they in fact exist, might play important roles in the mouse cell.

ACKNOWLEDGEMENTS

We thank Eric Schmader for excellent technical assistance, Dr. Tom Maniatis for supplying and Dr. Helen Coon for preparing HβG1, and Dr. Arthur Nienhuis for encouragement and helpful suggestions.

*To whom correspondence should be addressed.

†Current address: Bethesda Research Laboratories, P.O.B. 577, Gaithersburg, MD 20760, USA.

REFERENCES

1. Beggs, J. D. (1978) Nature (London) 275, 104-109.
2. Struhl, K., Stinchcomb, D. T., Scherer, S., and Davis, R. W. (1979) Proc. Natl. Acad. Sci. USA 76, 1035-1039.
3. Hsiao, C.-L., and Carbon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 3829-3833.
4. Wigler, M., Silverstein, S., Lee, L.-S., Pellicer, A., Cheng, Y.-C., and Axel, R. (1977) Cell 11, 223-232.
5. Bacchetti, S., and Graham, F. L. (1977) Proc. Natl. Acad. Sci. USA 74, 1590-1594.
6. Maitland, N. J., and McDougall, J. K. (1977) Cell 11, 233-241.
7. Mulligan, R.C., Howard, B.H., and Berg, P. (1979) Nature 277, 108-114.
8. Hamer, D. H., Smith, K. D., Boyer, S. H., and Leder, P. (1979) Cell 17, 725-735.
9. Anderson, W. F., Killos, L., Sanders-Haigh, L., Kretschmer, P. J., and Diacumakos, E. G. (1980) Proc. Natl. Acad. Sci. USA 77, 5399-5403.
10. Capecchi, M. R. (1980) Cell 22, 479-488.
11. Cline, M. J., Stang, H., Mercola, K., More, L., Ruprecht, R., Browne, J., and Salser, W. (1980) Nature 284, 422-425.
12. Meselson, M., and Yuan, R. (1968) Nature 217, 1110-1114.
13. Bolivar, F., Rodriguez, R. L., Green, P. J., Betlach, M. C., Heyneker, H. L., Boyer, H. W., Crosa, J. H., and Falkow, S. (1977) Gene 2, 95-113.
14. Kaufman, R. E., Kretschmer, P. J., Adams, J. W., Coon, H. C., Anderson, W. F., and Nienhuis, A. W. (1980) Proc. Natl. Acad. Sci. USA 77, 4229-4233.
15. Enquist, L. W., Vande Woude, G. F., Wagner, M., Smiley, J. R., and Summers, W. C. (1979) Gene 7, 335-342.
16. Kretschmer, P. J., Kaufman, R. E., Coon, H. C., Chen, M.-J., Geist, C. E., and Nienhuis, A. W. (1980) J. Biol. Chem. 255, 3204-3211.
17. Dagert, M., and Ehrlich, S. D. (1979) Gene 6, 23-28.
18. Lawn, R. M., Fritsch, E. F., Parker, R. C., Blake, G., and Maniatis, T. (1978) Cell 15, 1157-1174.

19. Lewin, B. (1974) Gene Expression-2. Eucaryotic Chromosomes, p. 7, John Wiley & Sons, London.
20. Hanahan, D., Lane, D., Lipsich, L., Wigler, M., and Botchan, M. (1980) Cell 21, 127-139.
21. Lusky, M., and Botchan, M. (1981) Nature 293, 79-81.
22. Huttner, K. M., Scangos, G. A., and Ruddle, F. H. (1979) Proc. Natl. Acad. Sci. USA 76, 5820-5824.
23. Hsiung, N., Warrick, H., deRiel, J. K., Tuan, D., Forget, B. G., Skoultchi, A., and Kucherlapati, R. (1980) Proc. Natl. Acad. Sci. USA 77, 4852-4856.
24. Perucho, M., Hanahan, D., and Wigler, M. (1980) Cell 22, 309-317.
25. Hirt, B. (1967) J. Mol. Biol. 26, 365-369.
26. Chang, A. C. Y., Lansman, R. A., Clayton, D. A., and Cohen, S. N. (1975) Cell 6, 231-244.
27. Sutcliffe, J. G. (1979) Cold Spr. Harb. Symp. Quant. Biol. XLIII, 77-90.
28. Wagner, M. J., Sharp, J. A., and Summers, W. C. (1981) Proc. Natl. Acad. Sci. USA 78, 1441-1445.