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Fate of exogenous recombinant plasmids introduced into mouse and human cells

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ABSTRACT

We have constructed a number of plasmids selectable in both E. coli and mouse or human cells. Human DNA sequences were inserted and the recombinant plasmids were used to transfect either mouse or human cells by the Ca-phosphate precipitation technique. We have observed that: (i) competent cells uptake large amounts of plasmid DNA; (ii) input plasmids persist in trasformed mammalian cells as free unreplicating circular molecules for up to 20 generations; such persistence does not depend on the presence of selective markers; (iii) plasmids incorporated into mouse L-cells undergo widespread rearrangements (in the absence of replication) entailing mostly deletions of both human and bacterial sequences which yield smaller products; the latter appear to be more stable in a subsequent transformation cycle. Surprisingly such rearrangements are almost totally absent in transformed human KB-cells. This property of human KB-cells may prove useful for the development of a vector apt at cloning and expressing human DNA sequences. Unlike what has been observed in yeast, no "autonomously replicating sequence" can be detected in mammalian cells by randomly cloning human DNA sequences into a selectable plasmid and screening for an increased transformation efficiency.

INTRODUCTION

DNA mediated gene transfer has been a useful tool to study the function and regulation of eukaryotic genes, the replication of animal viruses as well as to clone single mammalian genes. The Ca-phosphate precipitation technique is one of the most efficient methods to introduce DNA into cultured mammalian cells; some aspects of the process by which mammalian cells take up exogenous DNA and integrate it in the genome have already been elucidated (1-5).

Different DNA molecules coprecipitated with calcium-phosphate have been found integrated into the cellular genome in adjacent position (3). On

the basis of this evidence it has been proposed that, in the host cells, linear DNA fragments are ligated together into long linear molecules that are in turn the precursors of the integrated form (3,4). Very little is known about the fate of circular DNA molecules after transfection particularly regarding their expression, their physical modifications and the mechanisms that control the free vs integrated state. It is conceivable that these molecules might also persist in an extrachromosomal state and be inherited in a non-mendelian fashion when carrying a suitable origin of replication (4,5). In effect recombinant plasmids carrying viral replication genes have been shown to persist as freely replicating circular molecules, for several cellular generations (6-11). Such replicating molecules undergo various types of rearrangements among which deletions and point mutations are predominant. The aim of this work was to see whether circular DNA molecules, devoid of viral origins, could persist extrachromosomally for several generations after transformation in the absence of carrier DNA. We also wanted to determine whether the presence of human DNA sequences on these plasmids could influence their persistence in trasformed cells and/or the extent of rearrangement. For this purpose we inserted human DNA sequences into the selectable plasmids pOE3 (a derivative of pAT153 bearing the HSV tk gene and the Tn5 DNA fragment coding for G418 antibiotic resistance) and we analyzed the persistence and the modifications of the recombinant plasmids when introduced in human and mouse cells by the Ca-phosphate precipitation technique in absence of any carrier DNA.

MATERIAL AND METHODS

Cell culture and selective media

Mouse L-cells deficient in thymidine kinase (Ltk$^-$), a gift from Diane Robins (Columbia University), were grown in Dulbecco's modified Eagle's medium containing antibiotics (DMEM) and supplemented with 10% calf serum. Human KB-cells were grown in DMEM supplemented with 10% fetal calf serum. Ltk$^+$ transformants were selected in DMEM supplemented with 10% calf serum, 15 μg/ml hypoxantine, 1 μg/ml aminopterin and 5 μg/ml thymidine (HAT) (12). Selection for G418 resistants was done in growth medium containing 400 μg/ml

of G418 antibiotic (13). Samples of antibiotic G418 were kindly provided by Schering Corporation.

Plasmid construction

The plasmids used in this work were derived from plasmid IPB1 (kindly provided by Diane Robins, Columbia University) which carries the bacterial Tn5 neo^r gene (14), conferring the resistance to neomycin, inserted into the BglII site of HSV tk gene (15) The construction of the recombinant plasmds is described in Figure 1. IPB1 plasmid was partially digested with EcoRI, the 5.9 Kb fragment containing the neo^r gene was purified by low melting agarose gel (16) and ligated with T4 DNA ligase to EcoRI digested pAT153. E. coli strain MC1061 cells (17) were transformed with the ligation mixture to ampicillin and neomycin resistance; clones containing the 5.9 Kb fragment inserted into pAT153 (pOE1) were isolated and used to prepare plasmid DNA. pOE1 DNA, partially digested with SalI, was then treated with S1 nuclease, circularized with T4 DNA ligase and used to transform MC1061 cells to tetracycline and neomycin resistance. One selected clone, containing a plasmid of 8.6 Kb and designated pOE2, was further characterized. This plasmid differs from the parental pOE1 for the deletion of 900 bp (including the SalI site) in the Tn5 sequence downstream the neo^r gene. pOE2 was partially digested with BamH1, the ends were then filled in with T4 DNA polymerase to remove the BamH1 site; after ligation the reaction mixture was used to transform MC1061 cells to tet^r. The resulting plasmid pOE3, which confers the ampicillin, tetracycline and neomycin resistances to bacteria, and the G418 resistance to mammalian cells, was chosen as vector. The single BamH1 site in the tet^r gene was used to insert human DNA fragments.

ptk2a is a derivative of ptk2 (15) constructed by inserting the HSV PvuII fragment containing the tk gene, into the EcoRI site of pAT153 rendered blunt end with the pol I Klenow fragment.

pOE8 is a derivative of ptk2a obtained by replacing the BglII-SmaI fragment of the tk gene with the BglII-HincII DNA segment of Tn5 which contains the entire neo^r bacterial gene. pOE8 has three unique restriction sites: BamHI, SalI and HindIII in the tet^r gene.

DNA transfection of mammalian cells

Mouse and human cells were transfected with recombinant plasmids by the Ca-phosphate precipitation technique of Graham and van der Eb (18) as modified by Wigler et al. (19). L-cells (10^6/100 mm dish) and KB-cells ($2x10^5$/dish), plated the day before, were treated with plasmid DNAs at the concentration of 2 and 10 μg per dish respectively, unless otherwise specified in the text. No carrier DNA was used. The cells were exposed to the precipitated DNA for approximately 20 hours, then the medium containing DNA was removed, the cells washed with PBS without Ca^{++} and Mg^{++} ions and fresh culture medium was added. The following day selective pressure was applied; the selective medium was changed every 2-3 days and about 3 weeks later macroscopic colonies were counted. In the cotransformation experiment two plasmids were precipitated together and the cells were treated with the DNAs as described above.

Low molecular weight DNA preparation

Colonies, from two plates of transformed cells, were trypsinized, pooled and expanded in selective medium to approximately $2x10^7$ cells. Low molecular weight DNAs were isolated from dishes of cultured cells using the Hirt extraction procedure (20).

High molecular weight DNA preparation

High molecular weight chromosomal DNA was prepared from cultured cells as described by Maniatis et al. (21).

Bacterial transformation and plasmid DNA isolation

E. coli strain HB101 was transformed by the calcium chloride procedure of Dagert and Ehrlich (22). Large scale isolation of plasmid DNAs was performed according to the procedure of Birboim and Doly (23) as modified by Ish-Horowicz and Burke (24). Supercoiled DNAs were then purified by caesium chloride density gradient. Low molecular weight DNA isolated from human and mouse cells was used to transform cells of E. coli strain MC1061 by the calcium chloride-rubidium chloride procedure (25). The efficiency of transformation routinely obtained with this method was about $5x10^7$ trasformants per μg of pAT153 supercoiled DNA. Usually one tenth of each Hirt supernatant (corresponding to 10^6 cells) was used in each

transformation. Ampicillin resistant colonies were picked up and plasmid DNAs were prepared from 10 ml overnight cultures by the alkaline lysis method (24) followed by RNAse treatment.

Agarose gel electrophoresis and filter hybridization

Low molecular weight DNA, either intact or digested with restriction enzymes, was electrophoresed through 0.8% agarose gel in TAE buffer (40 mM Tris-acetate pH 7.5, 2 mM EDTA) containing 0.5 μg/ml ethidium bromide. The gels were treated twice with 0.25 M HCl for 15 min and washed in H_2O; the DNA was then denaturated by two 30 min treatments in 0.5 M NaOH/1 M NaCl and transferred to nitrocellulose filter according to the procedure of Wahl et al. (26). Filters were hybridized with the labelled probe (about 10^5 cpm/cm^2) for 18-20 hours at 42°C. Plasmid DNAs were labelled by nick-translation at specific activity greater than $2x10^8$ cpm/μg using (α-^{32}P) dCTP (3000 Ci/mmole, Amersham).

RESULTS

Transformation of murine and human cells with pOE3 and derivatives

The construction of plasmid pOE3 is described in Materials and Methods and its structure is shown in Figure 1. First we compared the dependence on DNA concentration of transformation to G418 resistance in murine L-cells and human KB-cells using pOE3 DNA. The results shown in Figure 2 evidence a markedly different response by the two types of cells: while KB-cells are not significantly transformed up to 2 μg/plate and then show a linear response, the dose-response curve for L-cells is linear between 0.1 and 2 μg/plate. In all our subsequent experiments we used limiting DNA concentrations (2 μg and 10 μg for L-cells and KB-cells respectively). Recombinant plasmids were constructed as follows: a partial Sau3AI digest of DNA from human HL-60 cells was prepared and, after size fractionation, molecules greater than 6 Kb were inserted into the unique BamHI site of pOE3. E. coli cells were then transformed with the ligation mixture and 20 pasmids containing inserts ranging between 4 and 14 Kb were selected and used to transform L-cells and KB-cells to G418 resistance. As shown in Table I, which reports the results of five representative plasmids, transformation

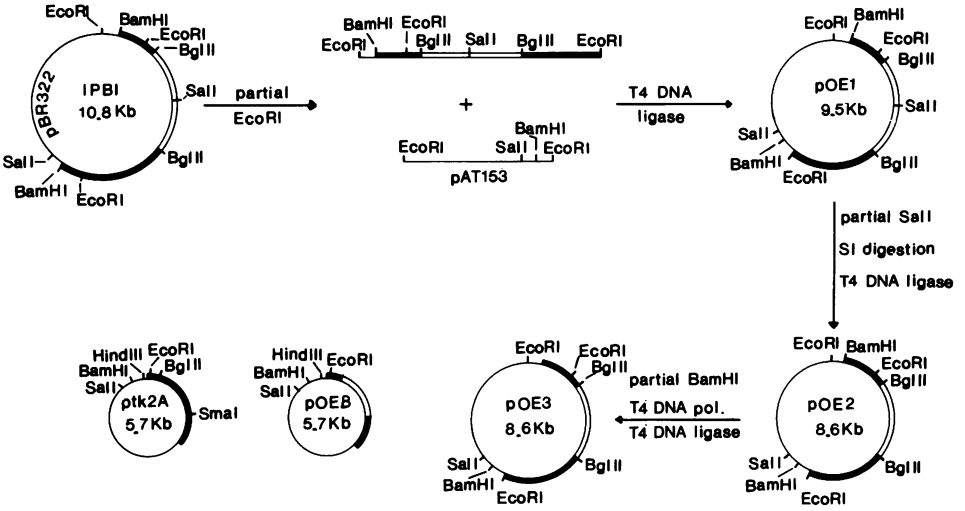

Fig. 1 - Construction of pOE1, pOE2 and pOE3 plasmids from IPB1. The structures of ptk2a and pOE8 are also shown. For details see Material and Methods (——) pAT153 or pBR322 sequences; (▬) HSV sequences; (▭) Tn5 sequences.

frequencies range between 0.5×10^{-4} and 12×10^{-4} with no significative differences between the two types of cells as compared to a control (vector alone) value of 3×10^{-4}. This result can be explained by assuming that both

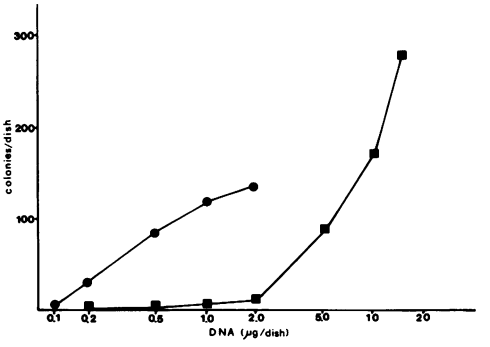

Fig. 2 - Dose-response curve for transformation of mouse L-cells and human KB-cells to G418 resistance by pOE3 DNA. KB-cells (■) and L-cells (●) (10^6 cells/plate) were transfected without carrier DNA as described in Materials and Methods. Colonies were scored after 3 weeks in selective medium (400 µg/ml G418).

TABLE I: Transformation frequencies of recombinant plasmids in mouse L-cells and human KB-cells[a].

Plasmid[b]	DNA insert size (Kb)	Transformation frequencies[c] ($x10^{-4}$)	
		L-cells	KB-cells
pOE3	0	2.4	3.7
pOE6-1	9.1	12.0	12.0
pOE6-2	13.9	7.7	3.4
pOE6-8	8.0	3.4	0.5
pOE6-12	7.2	2.7	1.2
pOE6-15	8.2	0.7	0.8

[a] Mouse L-cells (10^6/100 mm dish) were transfected with 2 µg of plasmid DNA as described in Material and Methods. Human KB-cells ($2x10^5$/100 mm dish) were transfected with 10 µg of plasmid DNA.

[b] pOE6-1, pOE6-2, pOE6-8, pOE6-12 and pOE6-15 plasmids were constructed by inserting human DNA fragments obtained by a partial Sau3A digest into the BamH1 site of pOE3.

[c] The frequency was calculated from the number of colonies scored after 3 weeks in selective medium.

L-cells and KB-cells pick up large amounts of the precipitated DNA and, in the presence of carrier DNA, efficiently integrate the uptaken sequences into their genome (1,3). Since a single integrated copy of the $G418^r$ gene is sufficient to give a resistant phenotype (13), it is impossible in our case to infer the presence and the number of extrachromosomal molecules from the frequency of transformation. In order to test whether circular DNA molecules can persist extrachromosomally following transformation in the absence of carrier DNA and whether such persistence is influenced by the presence of inserted human DNA sequences we directly analyzed the Hirt supernatants of the transformed cells for the presence of free circular molecules.

Analysis of the extrachromosomal DNA molecules in the transformed colonies

For each transformation experiment all the colonies growing in G418 were pooled and cultured under selective pressure for approximately 20 generations up to $2x10^7$ cells.The low molecular weight DNA was extracted and analyzed by Southern blotting with pOE3 probe. As shown in Figure 3, sequences hybridizing to pOE3 were observed in all transformed cultures and, when compared with the pattern of input plasmid, such sequences appear to

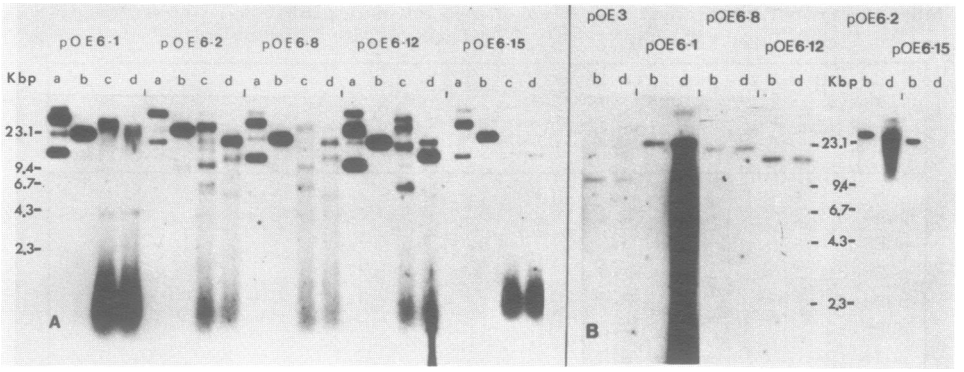

Fig. 3 - Southern blot analysis of low molecular weight DNAs from transformed mouse cells, panel (A), and human cells, panel (B). L-cells (10^6/dish) and KB-cells ($2x10^5$/dish) were transformed with 2 μg and 10 μg respectively, of the indicated recombinant plasmids. All the colonies yielded in two plates were pooled and grown up to $2x10^7$ cells. Low molecular weight DNA was extracted by the Hirt procedure and aliquots (1/5) were electrophoresed on 0.8% agarose gel: undigested (lanes c), digested with SalI, which cleaves our plasmids only once, (lanes d). As controls, 50 pg of the plasmids used in transformation were run: undigested (lanes a) and SalI digested (lanes b).

belong to plasmid-like molecules. The majority of such plasmids could be rescued in *E. coli* as amp^r plasmids and most of them expressed the neo^r gene. It is important to notice that the size of the plasmids rescued from transformed *E. coli* cells is identical to that observed in Hirt extract molecules by Southern blot analysis. The copy number per cell was estimated both from the intensity of the autoradiographic bands in Southern blots and from the number of amp^r bacterial colonies obtained in transformation. The two indipendent estimates were in reasonable agreement in all cases. This is a further indication that the molecules present in the Hirt extracts are in fact plasmids. The data of five recombinant plasmids and of the parental vector pOE3 are reported in Table II; the copy numbers range between 0.005 and 0.88. Subcloning of cultures or expansion of single colonies give progressively smaller copy numbers, down to non detectable levels. When L-cell colonies growing in G418 were diluted 10 fold and then grown to the same final number of cells ($2x10^7$) as in the previous experiment, the number

TABLE II: Free plasmid copy number in mouse L-cells and human KB-cells twenty generations after transfection[a].

Plasmid	Mouse L-cells copy number		Human KB-cells copy number	
	by hybridization analysis[b]	by bacterial rescue[c]	by hybridization analysis[b]	by bacterial rescue[c]
pOE3	N.D.	0.0074	0.064	0.027
pOE6-1	0.065	0.014	0.40	0.31
pOE6-2	0.42	0.15	0.21	0.084
pOE6-8	0.040	0.090	0.1	0.2
pOE6-12	0.65	0.88	0.005	0.016
pOE6-15	0.034	0.047	0.03	0.08

[a] Low molecular weight DNA was prepared as described in the legend to Figure 3.

[b] The estimation of the copy number was based on the intensity of the bands present in the Hirt supernatants with respect to the control (Figure 3).

[c] *E. coli* strain MC1061 was transformed as described in Material and Methods with one tenth of the Hirt supernatant. The copy number was calculated by comparison of the number of *amp*^{r} colonies yielded by the Hirt supernatant with that obtained with 20 pg of the same plasmid used in transfection.

of plasmids per cell decreased to undetectable values. Also, after growing single colonies up to $2x10^7$ cells under selective pressure, an extremely low level of free plasmids (copy number less than 0.005) was detected (data not shown). These data suggest that the extrachromosomal plasmids with or without human DNA inserts are mostly, if not all, input unreplicated DNA. We will further address this point in the last section.

Analysis of the integrated DNA molecules in the transformed colonies

The low level of free plasmids detected in G418^r cells after more than 20 cell generations (less than 1 copy per cell) indicates that the maintenance of the G418^r phenotype must be due to chromosomal integration of *neo* sequences.

High molecular weight DNA extracted from three indipendent G418^r L-cell colonies, transfected with pOE6-1 (carrying a 9.1 Kb human DNA insert), was analyzed for the presence of *neo* sequences. DNAs were digested with the restriction enzymes *KpnI* and *BglII* and hybridized on a Southern blot filter with the IPBI *BamHI* fragment containing the *neo* and *tk* sequences

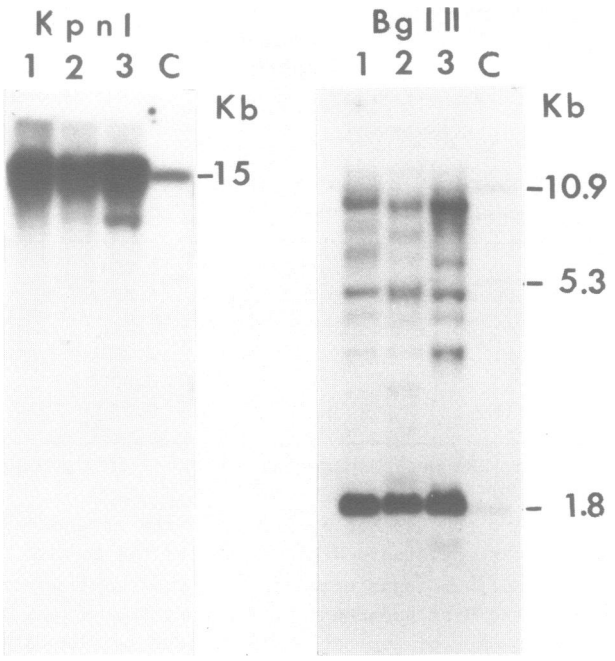

Fig. 4 - Southern blot analysis of the integrated pOE6-1 plasmid sequences in the DNA of three L-cells transformats. 10 µg of high molecular weight DNA were digested with restriction endonucleases, fractionated by electrophoresis on 0.8% agarose gel, and hybridized with the IPB1 BamH1 fragment containing the tk and neo sequences. Lanes 1,2,3: DNA extracted from the three indipendent L-cell lines and digested with the indicated restriction enzyme. Lanes C: 20 pg of pOE6-1 DNA digested with the indicated restriction enzyme.

(see Fig. 1). KpnI cleaves the pOE6-1 plasmid twice in the human sequences, while BglII recognizes three sites: two at the junctions between the neo and tk sequences and one in the human insert. As shown in Fig. 4, the three clones reveal a similar hybridization pattern with both enzymes and by the intensity of the band corresponding to the 1.8 Kb BglII fragment containing the neo sequences, we can estimate that between 5 and 10 copies are integrated in the genome. Among the fragments generated by BglII digestion only the 1.8 Kb band corresponds to a BglII pOE6-1 fragment. It is worth noticing that the restriction patterns of the three clones reveal a common set of well represented bands, probably generated by specific rearrangements

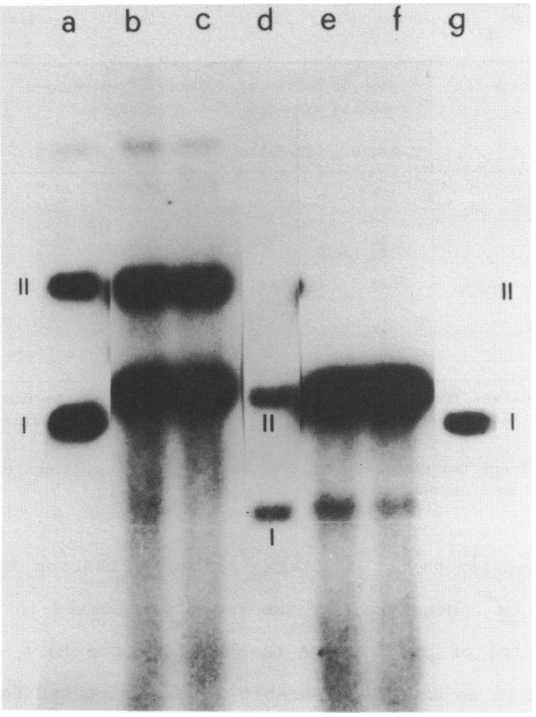

Fig. 5 - Southern blot analysis of Hirt supernatants of transformed mouse and human cells after MboI digestion. Left lanes: pOE8 DNA (10 μg) and pAT153 DNA (10 μg) were coprecipitated with Ca-phosphate and used to transform KB-cells ($2x10^5$/plate) to $G418^r$. Low molecular weight DNA was extracted and electrophoresed on 0.8% agarose gel: undigested (b) and digested with MboI (c). Right lanes: ptk2a DNA (0.2 μg) and pAT153 DNA (10 μg) were coprecipitated with Ca-phosphate and used to transform Ltk$^-$ cells (10^6/plate) to tk$^+$. Low molecular weight DNA was exctracted and treated as above: undigested (e) and digested with MboI (f). As control, pOE8 (a), pAT153 (d) and ptk2a (g) digested with MboI are shown.

occurring in the process of integration.

Cotrasformation with selectable and non selectable plasmids

The results reported in the previous sections suggest that the persistence of the free plasmids in the transformed cells is not due to the presence of neo^r and tk selectable genes. If this is the case then we would expect that also an unselectable plasmid like pAT153, containing only bacterial sequences, would be passively inherited after transformation. In order to test this hypothesis, we transfected L-tk$^-$ cells with pAT153

TABLE III: Rearrangement frequencies among recombinant plasmids extracted from L-cells and KB-cells[a].

Plasmid	Plasmid size (Kb)	Number of bacterial colonies examined		Number of rearranged plasmids		% Rearranged	
		L-cells	KB-cells	L-cells	KB-cells	L-cells	KB-cells
pAT-153	3.6	156	300	0	0	< 0.71	< 0.3
pOE3	8.9	18	10	11	1	61	10
pOE6-1	18.0	36	150	36	0	100	< 0.6
pOE6-2	22.8	28		28		100	
pOE6-8	16.9	22	14	18	2	81	14
pOE6-12	16.1	22	10	16	0	73	< 10
pOE6-1 HA[b]	14.6	12		5		42	
pOE6-1 HB[b]	10.2	12		0		< 9	

[a] E. coli strain MC1061 was transformed to amp^r with an aliquot of the Hirt supernatant. Single colonies were screened for the presence of neo^r and the extracted plasmids were analyzed by restriction enzymes.

[b] pOE6-1 and POE6-1 HB are two plasmids recovered in the Hirt supernatant of L-cells transformed with pOE6-1 and used in a second round of transfection.

together with a smaller amount of ptk2a, a plasmid bearing the HSV tk gene, and selected for tk^+ phenotype. As the results reported in Figure 5 show, intact DNA molecules of pAT153 were recovered in the Hirt supernatants of the transformants in an amount comparable to that reported for pOE3 in Table II.

A similar experiment was performed on KB-cells, with pAT153 and pOE8 as selectable plasmid, transforming for the ability to grow in G418. Also in this case the selected and non selected plasmids were present at similar levels after culture expansion (see Figure 5).

Rearrangements of input plasmids inside animal cells

As Figure 3 shows, large plasmids were usually not recovered intact from transformed L-cells, their size being invariably smaller than that of the input plasmid. Such effect seems to decrease in parallel with the plasmid size. As a matter of fact we did not observe a continuous decrease of sizes, but rather discrete species which appeared to represent some kind of final product: in fact, when these last forms were utilized for a second transformation cycle in L-cells, they seemed to be more stable. The data for a large number of plasmids are summarized in Table III. The frequency of rearrangements detected can be understimated because minor modifications such as small deletions and point mutations could escape our analysis.

Deletions, point mutations and complex rearrangements, have been detected with shuttle vector plasmids replicating in L-cells and in simian cells (27-29). It is remarkable that we can detect specific (non random) rearrangements in unreplicating molecules; such rearrangements, affect both bacterial and eukaryotic sequences (data not shown). Surprisingly rearragements were observed at a much lower extent in KB-cells (see Fig. 3 and Table III), where the majority of the input plasmids could be recovered essentially intact; in fact they maintained not only their original size, but also the same restriction and antibiotic resistance patterns. Thus mouse and human cells seem to differ markedly in their ability to rearrange exogenous DNA in the absence of replication.

The input plasmids do not replicate inside the cells

In order to determine whether the input plasmid DNA undergoes replication in transformed cells, the DNA in the Hirt supernatants was tested with MboI, an endonuclease that does not cut methylated DNA of plasmids grown in dam^+ bacteria, as are all our plasmids, while it would digest the same DNA after just one round of replication in mammalian cells. As Figure 5 shows, both the input DNA and the DNA extracted from the transformed cells were fully resistant to this enzyme. This result holds true for all 20 recombinant plasmids described in the first section (data not shown). This finding however is not sufficient to completely rule out an initial limited burst of replication in a fraction of the input plasmids. It is also possible that no replication was seen because none of the 20 recombinant plasmids tested contained a sequence (origin) conferring replicative capacity. To check these points we tested a much wider array of human chromosomal sequences, and adopted a more stringent criterion for replication after transfection i.e. the acquisition of resistance to DpnI restriction enzyme. This enzyme in fact cuts only methylated DNA at the $G^{me}ATC$ sequence and is therefore unable to digest DNA replicated in mammalian cells which lack A-methylase activity. Chromosomal human DNA was digested with either BamHI or HindIII and fragments were ligated into pOE8 DNA. After transformation of E. coli strain HB101 with the ligation mixture, 500 independent transformed colonies (amp^r, tet^s) were pooled, expanded in

liquid culture and the plasmids extracted. Such plasmid collection is composed of inserts that cover at least 2500 Kb of the human chromosome and should therefore contain a certain number of replication origins. Two types of experiments were performed: (i) KB-cells were transfected with the plasmid mixture in the absence of selective pressure and after 5 days Hirt supernatants were prepared from the whole cell population; (ii) KB-cells were transfected with the same plasmid mixture, colonies transformed to $G418^r$ were pooled, grown up to $2x10^7$ (about 20 generations) and Hirt supernatants prepared. Low MW DNAs before and after Dpn1 digestion, were then used to transform E. coli MC1061 and the number of amp^r colonies counted. In both types of experiments no amp^r colonies were observed with Dpn1 digested DNAs as opposed to $5x10^5$ and 10^5 colonies in undigested controls. Therefore no evidence of replication can be obtained with any of the above mentioned approaches.

DISCUSSION

The data presented in this paper indicate that transformed cells uptake large amount of DNA following transformation with the Ca-phosphate precipitation technique and maintain circular DNA molecules in an extrachromosomal state for up to 20 generations. Molecules present in the Hirt supernatant were analyzed by Southern blot (before and after Sal 1 digestion) and used to transform E. coli MC1061 strain to Amp^r or Neo^r. The two estimates of the number of molecules present in the Hirt supernatants, obtained independently by these methods, are in perfect agreement indicating that such molecules are in the form of circular plasmids. The total amount of unreplicated DNA recovered 20 generations after transformation divided by the number of initially transformed colonies yields a value of 2 pg for the amount of DNA incorporated into each transformed cell. This value can be understimated since Southern blots often show a smear of hybridizing material probably representing degraded DNA unable to transform E. coli.

While in human KB cells plasmid molecules present after 20 generations seem to be identical to the input ones, in mouse L-cells they appear widely rearranged regardless of whether they carry human DNA sequences or not. In

fact both the vector POE3 and its derivatives undergo rearrangements which yield smaller molecules. The fact that the size of the plasmid rescued from transformed E. coli cells is the same as that observed in Southern blots rules out possible artefacts due to amplification in E. coli. It is worth noticing that the frequency of rearrangement is to a certain extent related to the size of the transforming plasmid (see Tab. III). This could explain the observed relative stability of pAT153 and of other small plasmids (data not shown).

On the other hand the observed rearrangements are not random since each input molecule originates a distinct set of discrete rearranged molecules. The rearranged plasmids, on their turn, appear to be more stable in a second transformation (Tab. III) and their rearrangement frequency is less than that observed for the pOE3 vector, in spite of their bigger size. Altogether these data suggest that both size and sequence influence the stability of the transfected plasmids.

It is remarkable that we can detect specific rearrangements in unreplicating molecules; somewhat related observations have recently been reported for a pML-SV40 recombinant plasmid in monkey cells (28).

We have also analyzed the number and the state of plasmid molecules integrated into the genome of the transformed cells. From the restriction enzyme analysis of three indipendent G418^r L-mouse colonies it is evident that 5 to 10 plasmid copies are integrated in the genome. The restriction pattern obtained by digestion with BglII indicates that the integrated molecules have undergone several rearrangement events, before or during the integration process; infact only the 1.8 Kb fragment (carrying the neo sequences) is conserved intact in transformants. On the other hand the presence of a set of well represented bands shared by all three cellular clones suggest that specific rearrangements have taken place. This observation reminds the results reported for the unintegrated plasmids.

Mouse L-cells and human KB-cells differ in many respects: (i) they show a different dose-response kinetics for transformation to G418 resistance (see Figure 2), (ii) they are drastically different from the stand point of input plasmid rearrangements, KB-cells being almost devoid of

such activity. Several hypotheses can be made to explain the difference between L-cells and KB-cells: the two types of cells could use different enzymatic pathways for recombination or else in KB-cells plasmid DNA is more protected from the action of recombinative enzymes. In any case transformation in KB-cells is quite interesting particularly in view of the development of a shuttle vector and of its use in the study of gene structure and expression in human cells.

We have been unable to detect any evidence of autonomous replication of recombinant plasmids upon transfection into L-cells or KB-cells despite the fact that the great number of inserts tested should have insured the presence of an origin of replication in at least some of them (one origin of replication should be present every 50-100 Kb (30)). All these data make us wonder whether a functional origin of replication is just a DNA sequence or a more complex entity entailing both sequence and chromosomal structure which cannot be reproduced in a single plasmid molecule.

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