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Genetics

Direct transfer of cloned genes from bacteria to mammalian cells

(simian virus 40/DNA uptake/eukaryotic recombination)

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ABSTRACT **Induction of a virus infection by cloned simian virus 40 DNA was chosen as a test system to detect transfer of genes from bacteria to cultured mammalian cells. *Escherichia coli* cells containing a recombinant plasmid with three tandem inserts of simian virus 40 DNA were able to infect CV-1 monkey cells under various conditions. The gene transfer was resistant to DNase I and therefore seems not to occur via free DNA but most likely via uptake of whole bacteria, followed by release of plasmid DNA and generation of infectious circular simian virus 40 DNA in a recombination–excision process. Spontaneous transfer was found to be infrequent, 4×10^9 bacteria yielding one infection per 10^7 monkey cells. The frequency was greatly increased by adding bacteria as a calcium phosphate coprecipitate or by fusion of lysozyme-treated bacteria (protoplasts) with monkey cells in the presence of polyethylene glycol. With the latter technique, 10^4 protoplasts gave rise to one infection per 15 monkey cells. Experiments with other cell lines of human, monkey, and mouse origin, and also with bacteria harboring another recombinant plasmid, indicate that DNA transfer from bacteria to mammalian cells is a general phenomenon.**

For more than 30 years it has been known that DNA can be transferred from one organism to another, thereby changing the genetic constitution of the recipient (1). In bacteria there exist various mechanisms for gene exchange among closely related species (for review see ref. 2). Gene transfer between unrelated species, or even between prokaryotes and eukaryotes, is rarely observed. A well-documented case of the latter category is the induction of crown gall tumors in higher plants by *Agrobacterium tumefaciens*; in this case a bacterial plasmid is taken up and expressed by the plant cell (for review see ref. 3). Recently, Hinnen and Hohn (4) were able to transform yeast cells by treating them with bacteria containing cloned yeast genes. Mammalian cells in culture treated with purified DNA can take up and express viral (5, 6) or cellular (7, 8) genes. Similarly, virus infections and tumors can be induced by injection of viral DNA into whole animals (9). Nevertheless, direct transfer of cloned genes from bacteria to mammalian cells has not been detected so far (10, 11). In order to monitor such a gene transfer, an extremely sensitive test system was devised in which bacteria harboring cloned simian virus 40 (SV40) DNA induced a viral infection in cultured monkey cells. This method is superior to transformation of recipient cells by a drug-resistance marker because it does not depend on stable integration of the transferred DNA and because the background of false positives is zero. The only difficulty involves the generation of infectious SV40 from cloned viral DNA. Purified recombinant plasmid or phage DNA containing one full copy of polyoma or SV40 DNA cannot induce a viral infection (refs. 10–12; this paper). However, infectious viral DNA is created within the host cell if linearly integrated viral DNA contains a terminal redundancy of the type xxabcdeabxx, where a–e denotes viral sequences and x denotes flanking nonviral DNA. Such head-to-tail duplications are found in most cell lines transformed by SV40 (13–15) or polyoma virus (16), in adenovirus–SV40 hybrid viruses (ref. 17; Y. Gluzman and J. Sambrook, personal communication), and in recombinant phages or plasmids containing dimer inserts of viral DNA (11, 12). For the transfer experiments described in this paper, bacteria containing a recombinant plasmid with three SV40 DNA molecules ligated in a head-to-tail fashion were used. This plasmid was a very good substrate for the recombination–excision system of the monkey host cell. The low frequency of spontaneous gene transfer could be dramatically increased either by offering the plasmid-harboring bacteria in a calcium phosphate coprecipitate, a procedure known to increase the uptake of purified DNA (6), or by treating bacterial protoplasts and monkey cells with polyethylene glycol which presumably resulted in membrane fusion and hybrid cell formation (for review see refs. 18 and 19). Experiments with other mammalian cell lines and with another recombinant DNA indicate that the observed gene transfer is generally valid and may be exploited to screen large numbers of bacterial colonies for the expression of cloned DNA in eukaryotic cells.

MATERIALS AND METHODS

Growth of Mammalian Cells and SV40. Cell lines used were kidney cell lines CV-1 and BSC-1 from the African green monkey, human carcinoma line HeLa, diploid human fibroblast line WI-38, and mouse fibroblast line 3T6. The tissue culture medium for all cells was Dulbecco's modified Eagle's medium containing 2.5% fetal calf serum, 2.5% calf serum, 100 units of penicillin per ml, and 100 μg of streptomycin per ml (GIBCO). Unless otherwise stated in *Results*, tissue culture medium was regularly supplemented with 100 μg of kanamycin per ml (Sigma) during and after treatment with bacteria. In experiments with long incubations, tissue culture medium was renewed every 3–4 days. SV40 growth, plaque assays, viral DNA preparations, and immunofluorescence assays were carried out as described (20).

Calcium phosphate coprecipitates of DNA or bacteria were added to tissue culture cells as described (7); 4 hr later cells were shocked with 15% (vol/vol) glycerol (21), rinsed with Tris-buffered saline (22), and further incubated in tissue culture medium.

Recombinant DNAs and Bacteria. Recombinant plasmids between pBR322 (23) and SV40 DNA were constructed by standard cloning procedures (24, 25). All work involving pBR322-SV40 recombinant plasmids was done under conditions conforming to the standards outlined in the National Institutes of Health Guidelines for Recombinant DNA Research.

Escherichia coli K-12 HB101 bacteria containing recombinant plasmids were grown in Luria broth at 37°C to a density of 2×10^8 cells per ml, pelleted by centrifugation at $5000 \times g$ for 10 min, and resuspended in a small volume of either tissue

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Abbreviations: SV40, simian virus 40; bp, base pairs; T antigen, large tumor antigen.

culture medium for direct application, or 50 mM Tris·HCl (pH 8) for calcium phosphate coprecipitation, or 20% (wt/vol) sucrose/0.1 M Tris·HCl (pH 8) for preparation of protoplasts. For several experiments (see *Results*) plasmid DNA was selectively amplified with chloramphenicol (Sigma; ref. 26). For the experiments in which no kanamycin was added to the tissue culture medium, bacteria were pelleted from the aqueous phase after shaking with 1/5th vol of chloroform or diethylether, or after addition of 2 vol of cold ethanol. They were then resuspended in Tris-buffered saline, centrifuged again, and resuspended in the appropriate solution as described above. Bacterial protoplasts (27) were slowly diluted with 3 vol of tissue culture medium without serum containing 7% (wt/vol) sucrose, 10 mM $MgCl_2$, and 0.2 μg of DNase I per ml and incubated for 10 min at room temperature before addition to tissue culture plates (DNase I treatment did not decrease the infectivity of protoplasts and abolished the viscosity of some preparations in which a small fraction of bacterial cells had lysed).

Bacteria to be treated with DNase I were suspended in tissue culture medium at 10^9 cells per ml. A 1/20th vol of DNase I [Worthington; 1 mg/ml in "enzyme dilution buffer": 20 mM Tris·HCl, pH 7.6/50 mM NaCl/1 mM dithiothreitol/100 μg of bovine serum albumin per ml in 50% (vol/vol) glycerol; stored at −20°C without loss of activity for several months] was added and the sample was incubated at room temperature for 15 min. DNase I was found to remain fully active throughout exposure of CV-1 cells to bacteria as assayed by gel electrophoresis of plasmid DNA incubated with samples of tissue culture medium.

Polyethylene Glycol Treatment. Protoplasts from 10^9 bacteria were added to one 60-mm petri dish with slightly subconfluent mammalian cells. The petri dishes were placed onto the flat bottom of swing-out buckets of an MSE Cool-Spin 6 × 1 liter rotor, and protoplasts were centrifuged onto the cell monolayer (3000 rpm for 5 min at room temperature). The clear supernatant was immediately removed and 2.5 ml of 47.5% polyethylene glycol [an autoclaved mixture of 95 g of molten polyethylene glycol 1000 (British Drug Houses, Poole, England) and 100 ml of Tris-buffered saline; stored at room temperature] were added, distributed over the plate by tilting, and then removed after 90 sec. WI-38 cells were treated with 42.5% polyethylene glycol because higher concentrations were found to be toxic for them. Residual polyethylene glycol was immediately dissolved in 4 ml of Tris-buffered saline by agitation for 1 min. After two more rinses with Tris-buffered saline, cells were incubated in fresh tissue culture medium.

RESULTS

Construction of Recombinant Plasmids. An SV40 DNA triple insert clone was constructed by ligating a 10-fold excess of *Bam*HI-digested SV40 wild-type DNA to *Bam*HI-digested pBR322 plasmid DNA. HB101 bacteria treated with the ligated DNA were selected for ampicillin resistance and screened for the presence of SV40 DNA. One of the analyzed bacterial colonies, which will be referred to as pBSV-3x, showed very strong hybridization to ^{32}P-labeled SV40 DNA. Plasmid DNA extracted from this clone was much larger than the DNA extracted from clones that contain one molecule of SV40 DNA linked to pBR322 (Fig. 1*a*). Restriction of this plasmid with the enzyme *Bam*HI and analysis of the fragments by agarose gel electrophoresis revealed a strong band in the position of linear SV40 wild-type DNA and a band about one-third as strong in the position of linear pBR322 DNA. Restriction by *Eco*RI yielded two bands of about equal intensity, one the size of linear SV40 wild-type DNA [5224 base pairs (bp)] and one of about 4800 bp (Fig. 1*a*, lane 6). Most likely, the upper band is linear

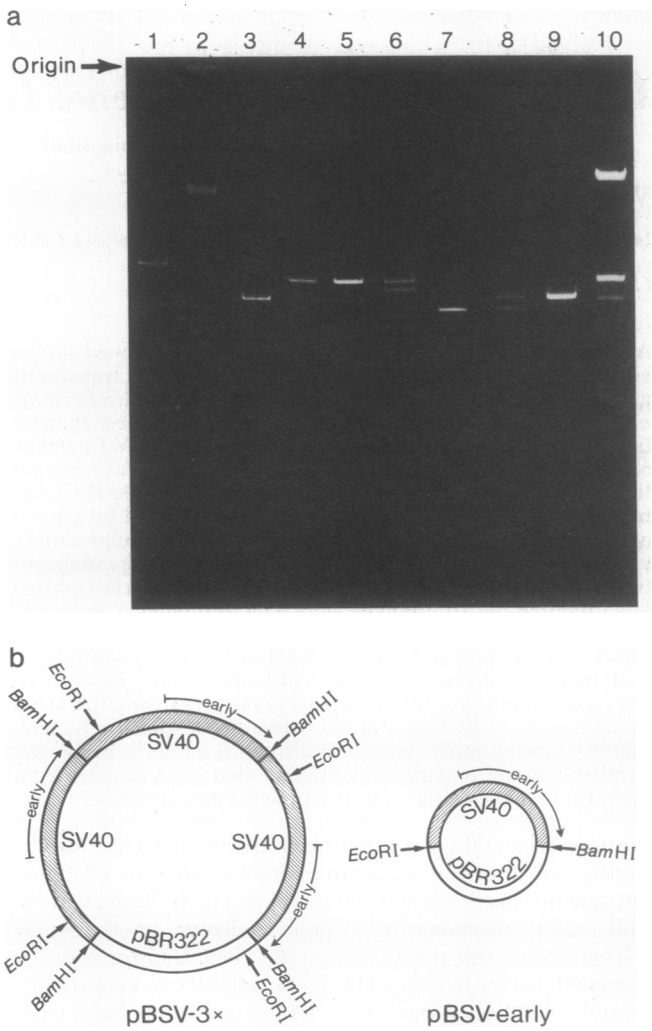

FIG. 1. Analysis and structure of pBR322-SV40 recombinant plasmids. (*a*) Plasmid DNA was extracted from bacteria and analyzed by restriction digestions and agarose gel electrophoresis. Lanes: 1, recombinant plasmid containing one copy of SV40 DNA integrated in the *Bam*HI site (uncleaved); 2, plasmid (pBSV-3x) containing three tandem copies of SV40 DNA integrated at the *Bam*HI site (uncleaved); 3, pBR322 cleaved with *Bam*HI (4362 bp; ref. 28); 4, pBSV-3x cleaved with *Bam*HI; 5, SV40 DNA cleaved with *Bam*HI (5224 bp; ref. 29); 6, pBSV-3x cleaved with *Eco*RI; 7, pBR322 cleaved with *Bam*HI and *Eco*RI (large fragment, 3987 bp); 8, plasmid (pBSV-early), which has the large *Bam*HI/*Eco*RI fragment of pBR322 linked to the large *Bam*HI/*Eco*RI fragment of SV40 (cleaved with *Bam*HI and *Eco*RI); 9, SV40 DNA cleaved with *Bam*HI and *Eco*RI (large fragment, 4478 bp; small fragment, 746 bp); 10, bacteriophage λ DNA cleaved with *Eco*RI and *Hin*dIII. (*b*) Structure of the recombinant plasmids pBSV-3x and pBSV-early as deduced from the above analysis.

SV40 DNA (two fragments per recombinant plasmid) and the lower band is a mixture of two different fragments, each containing a portion of the SV40 sequence and a portion of the pBR322 sequence. It was concluded that three molecules of SV40 are present as tandem repeats in the recombinant plasmid (Fig. 1*b*). A photographic negative of the ethidium bromide-stained gel was scanned and gave an intensity ratio of 1.00:0.95 for the upper/lower bands corresponding to a molar ratio of 2.00:2.08. This is compatible with the above conclusion. Another plasmid (referred to as pBSV-early) was also constructed from the large SV40 DNA fragment found after *Eco*RI and *Bam*HI double digestion and the large fragment of pBR322 generated by the same digestion (Fig. 1*a*, lanes 7–9; Fig. 1*b*). The structure

of both recombinant plasmids was confirmed by further restriction digestions (data not shown).

Biological Activity of pBSV-3x DNA and pBSV-Early DNA. Purified DNA from the pBSV-3x clone as well as from the pBSV-early clone were added to CV-1 monkey cells as a calcium phosphate coprecipitate. Both DNAs induced SV40 large tumor antigen (T antigen) production in 2–5% of the treated monkey cells as measured by indirect staining with fluorescent antibody. In addition, pBSV-3x DNA, but not pBSV-early DNA, was capable of initiating a viral infection of the treated cells, similar to the results obtained with cloned dimers of polyoma DNA (11, 12). The pBSV-3x clone, therefore, was chosen to test whether cloned SV40 DNA could be transferred directly from bacteria to monkey cells and thereby initiate a viral infection.

SV40 Infection Induced by Treatment with pBSV-3x Bacteria. pBSV-3x bacteria were plated on Luria broth agar without antibiotics; therefore, there was no selective pressure for maintenance or amplification of the plasmid. Three colonies of 1 mm diameter were pooled and serially diluted in Luria broth. Each sample was coprecipitated with calcium phosphate and added to a 60-mm plate with CV-1 monkey cells. In addition to testing colonies, a liquid culture of pBSV-3x grown under nonselective conditions was serially diluted and added as a calcium phosphate coprecipitate. Viral infections led to confluent cell destruction after 9–14 days. In order to exclude nonspecific cytopathic effects, fresh CV-1 cells were exposed to culture medium from such plates. High titers of SV40 virus were invariably detected by the immunofluorescence assay for SV40 T-antigen. As can be seen in Table 1, as little as 1% of one bacterial colony or 1 μl of a liquid culture (2×10^5 bacteria) was sufficient to induce a virus infection.

In order to find out if pBSV-3x bacteria were infectious even in the absence of procedures to enhance uptake, and also to find the conditions for maximal infectivity, various conditions were tested (Table 1). In summary, pBSV-3x bacteria were always infectious, although there were large differences in efficiency depending on the procedures used. In a "minimal treatment" (i.e., bacteria grown under nonselective conditions, pelleted, added to cultured monkey cells, and rinsed off after 4 hr at 37°C) 4×10^9 bacteria (pelleted from 20 ml of a liquid culture) could induce one viral infection per two 100-mm plates containing 5×10^6 monkey cells each. A much more efficient SV40 gene transfer was obtained if bacteria were added in a calcium phosphate coprecipitate as mentioned above or by polyethylene glycol treatment of bacterial protoplasts pelleted onto CV-1 monkey cells.

It was also demonstrated that this gene transfer was not dependent on kanamycin, which was usually present to prevent bacterial growth. pBSV-3x bacteria killed with ether, chloroform, or ethanol were added to CV-1 cells (2×10^8 bacteria per 60-mm plate) in the absence of kanamycin. All three samples induced an SV40 infection.

In order to get quantitative information on the proportion of CV-1 cells infected with SV40 at high concentrations of bacteria, 36 hr after treatment, cells were fixed onto the tissue culture plate, SV40 T-antigen was stained with fluorescent antibody, and the proportion of positive nuclei was determined in a fluorescence microscope. Because rounded-off mitotic cells and dead cells can exert unspecific fluorescence, only typical nuclei containing black nonfluorescent nucleoli were scored. With this criterion, the background (false positives) was zero. For these experiments pBSV-3x bacteria had been treated with chloramphenicol to amplify the plasmids (26). This resulted in a 2-fold increase in fluorescent cell nuclei as compared to the results without amplification (data not shown). The most efficient method of gene transfer (6.6% of all nuclei were fluorescent) was obtained with polyethylene glycol-mediated fusion of protoplasts to monkey cells (see also Fig. 2*a*). DNase I clearly destroyed DNA infectivity in the reconstruction experiment but had little effect on whole pBSV-3x bacteria or protoplasts (Table 2; see also Table 1).

In order to compare immunofluorescence with infectivity, CV-1 cells treated with pBSV-3x protoplasts and polyethylene glycol were trypsinized. One portion was reseeded and assayed by immunofluorescence, and the other portion was diluted with untreated CV-1 cells and subjected to a plaque assay. The ratio of plaques to fluorescent nuclei was 1.1, as compared to 0.9 for an SV40 virus infection done in parallel, thus indicating that about every cell producing T-antigen (which is not dependent on SV40 wild-type DNA excision) also generated infectious viral DNA from the triplicated cloned SV40 DNA.

General Application. The SV40 "early" region can be expressed not only by the genuine monkey host cells but also by cells from other mammals (for review, see ref. 30). Therefore, it was possible to determine whether the observed gene transfer was a peculiarity of the CV-1 recipient cells. In addition to CV-1 cells, the following cells were tested: BSC-1 (another kidney cell line of the African green monkey), HeLa (a human cervix carcinoma line), WI-38 (a diploid human fibroblast line), and 3T6 (a mouse fibroblast line). The cells were treated with pBSV-3x protoplasts and polyethylene glycol. At 36 hr after treatment, cells were fixed onto the plates and SV40 T antigen was stained with antibody and detected by fluorescence microscopy. CV-1, BSC-1, HeLa, WI-38, and 3T6 cells had 6.6, 2.6, 5.2, 0.1, and 1% fluorescent nuclei, respectively (Fig. 2 *a–e*).

The pBSV-early clone, which has a recombinant plasmid with 85% of the SV40 genome linked to pBR322 (Fig. 1*b*), was also assayed. CV-1 cells treated with pBSV-early protoplasts and

Table 1. SV40 infection of CV-1 cells after various treatments with plasmid-harboring bacteria (pBSV-3x)

% of bacterial colony (1 mm diameter) used	0	0.1	0.3	1	3	10	30	100
Infectivity with calcium phosphate and glycerol shock	−	−	−	+	+	+	+	+
Volume (ml) of bacterial culture used	0	10^{-4}	10^{-3}	10^{-2}	10^{-1}	1	10	
Infectivity with:								
No extra treatment	−	−	−	−	−	−	+*	
DNase I (50 μg/ml)	ND	ND	ND	ND	ND	ND	+†	
Glycerol shock	−	−	−	−	−	+	+	
Calcium phosphate and glycerol shock	−	−	+	+	+	+	+	
Protoplasts and polyethylene glycol	−	−	+	+	+	+	+	

Each value is from one 60-mm plate of CV-1 cells. ND, not determined.

* The 60-mm plate was not infected, but 7 of 13 100-mm plates became infected.

† Of 14 100-mm plates, 6 became infected (however, none of 14 100-mm control plates treated with HB101 bacteria became infected).

Table 2. SV40 T antigen expression after various treatments with plasmid-harboring bacteria (pBSV-3x)

	Treatments				Immunofluorescence		
	DNase I (0.2 μg/ml)	DNase I (50 μg/ml)	Calcium phosphate and glycerol shock	Polyethylene glycol	Fluorescent nuclei*	Total nuclei†	Fluorescent nuclei, %
Experiments							
pBSV-3x bacteria	—	—	Yes	—	37	147	0.4
pBSV-3x bacteria	—	Yes	Yes	—	26	158	0.3
pBSV-3x protoplasts	—	—	Yes	—	65	160	0.6
pBSV-3x bacteria	—	—	—	Yes	24	178	0.2
pBSV-3x protoplasts	—	—	—	Yes	276	141	3.1
pBSV-3x protoplasts	Yes	—	—	Yes	673	159	6.6
pBSV-3x protoplasts	—	Yes	—	Yes	615	165	5.8
Controls							
HB101 bacteria	—	—	Yes	—	0	ND	0
HB101 mixed with 5 μg plasmid pBSV-3x DNA	—	—	Yes	—	436	151	4.5
HB101 mixed with 5 μg plasmid pBSV-3x DNA	—	Yes	Yes	—	0	ND	0

* Number per 4 × 4 mm area.
† Number per 0.5 × 0.5 mm area. ND, not determined.

polyethylene glycol gave rise to SV40 T-antigen production in 1.9% of the cells (Fig. 2*f*), thus demonstrating that SV40 gene transfer is not dependent on a peculiar feature of the pBSV-3x clone.

DISCUSSION

In this paper a direct transfer of cloned genetic information from bacteria to mammalian cells has been demonstrated. The process is resistant to the addition of DNase I, indicating that transfer via free plasmid DNA from lysed bacteria can at best play a minor role. It is conceivable that entry into the monkey cell is brought about by phagocytosis both in the spontaneous transfer and in the calcium phosphate-enhanced transfer. The centrifugation of bacterial protoplasts onto the cell monolayer followed by treatment with polyethylene glycol most likely results in fusion of bacterial and mammalian cell membranes (18, 19) and subsequent release of plasmid DNA into the cytoplasm of the recipient cell. The low efficiency of spontaneous transfer (one single infection event after treating 10^7 monkey cells with 4×10^9 pBSV-3x bacteria) can be greatly increased by using the calcium phosphate coprecipitation method and even further increased by the polyethylene glycol treatment of bacterial protoplasts and monkey cells (one infection per 15 cells with 10^4 protoplasts).

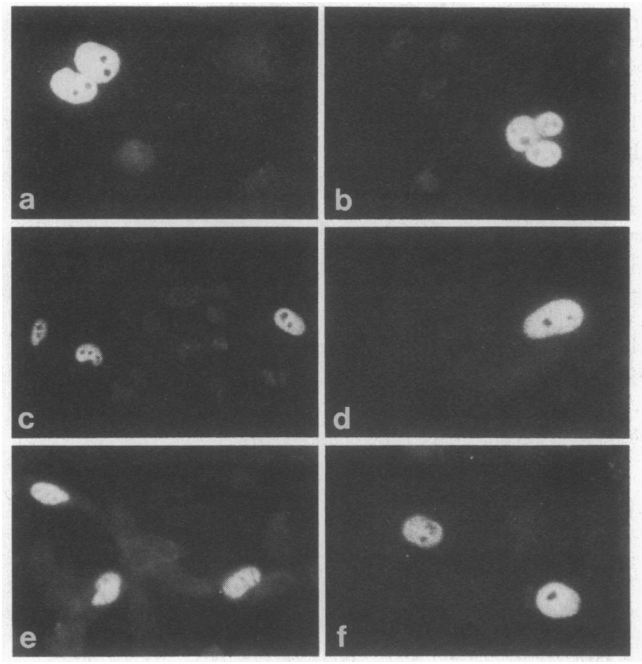

FIG. 2. SV40 T-antigen production in cells treated with bacteria harboring recombinant plasmids. Mammalian cells were treated with bacterial protoplasts (27) containing amplified plasmids (26) and polyethylene glycol. (*a–e*) Various cell lines treated with pBSV-3x protoplasts (*a*, CV-1; *b*, BSC-1; *c*, HeLa; *d*, WI-38; *e*, 3T6). (*f*) CV-1 cells treated with pBSV-early protoplasts. *a*, *b*, and *f*, ×200; *c*, ×180; *d* and *e*, ×270.

The system described here is capable of detecting very rare gene transfers. Once the pBSV-3x plasmid DNA was successfully transferred to the mammalian cell, an efficient recombination process generated infectious circular wild-type SV40 DNA from the triplicated SV40 insert. A recombination–excision step taking place already within the bacterial cell cannot be a significant source of infectious SV40 DNA because no SV40 wild-type DNA band is observed in gels overloaded with pBSV-3x plasmid DNA and because the infectivity of this plasmid on a molar basis approaches that of SV40 wild-type DNA (unpublished data). The recombination system creating infectious viral DNA seems to depend on terminal sequence redundancies (see Introduction). In adenovirus–SV40 hybrid viruses (17) and in pBR322–polyoma recombinant DNA (12) the extent of duplication was found to be correlated to the efficiency of wild-type virus DNA generation. Accordingly, the triple insert of SV40 DNA in pBR322 is a very good template for recombination–excision. About every cell expressing SV40 T antigen (which is not dependent on the presence of circular wild-type DNA) also produced infectious virus. This frequency of generation of infectious virus DNA from trimeric SV40 DNA is apparently much higher than that observed with dimeric polyoma DNA cloned in a bacteriophage λ vector (11) and may explain why these authors could not detect any polyoma infection after administration of massive doses of phage-harboring bacteria to mice. Nevertheless, in my experiments, 4×10^9 bacteria containing a total of at least 10^{11} plasmid molecules with the favorable array of three tandem SV40 DNA inserts were necessary to induce one SV40 infection in tissue culture cells. This is at most $1/10^9$ of the efficiency of infection with virus particles, in which an average of 100 particles induce one

infection (31). From these results and the results of others (10–12), it appears that work with cloned virus DNA is safer than work with the virus itself.

Cell transformation, which depends on stable integration of viral DNA into a host chromosome, was not assayed in these experiments but would be expected to occur at 0.01–1% of the induction of a lytic viral infection (31–33).

DNA transfer from bacteria to higher organisms seems to be an infrequent event but, considering the large population sizes and extended periods of time available, such a process probably does occur continuously in nature.

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