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New, Small Circular DNA in Transfected Mammalian Cells

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Circular DNA isolated by the Hirt procedure from transfected mammalian cells was examined by electron microscopy. Typically, the number of small (1- to 5-kilobase) DNA circles increased about fivefold even though DNA of larger size classes (5 to 15 kilobases) has been transferred. In one case, where extensive rearrangement of the transferred DNA was observed, the rearrangement products were cloned and analyzed. In most cases, however, no rearrangement could be detected, but the amount of small circular DNA was still increased. This effect was seen with two transfection procedures (erythrocyte ghost fusion and calcium phosphate precipitation) and with various combinations of transfecting DNA and recipient cell type. The origin of the new small circular DNA is discussed.

Transfection of DNA into mammalian cells is a widely used technique. The interest in such experiments is usually focused at expression of the transfected gene(s). The majority of analyses are performed at a long time (several days or weeks) after introduction of DNA. In the minority of cases where the situation at a short time after transfer is studied, electron microscopy of DNA is seldom employed. Examination by electron microscopy of the pool of extrachromosomal DNA in recently transfected mammalian cells is a field where few studies have been carried out.

In our electron microscopic studies of the replication in vivo of recombinants between polyomavirus and pBR derivatives (27), we consistently observed a large number of DNA circles considerably smaller than the one transfected (Fig. 1). To find out whether these were derived from the transfecting DNA or from the recipient cells, we analyzed the DNA from normal, untransfected cells. In agreement with earlier reports we found such molecules, although at considerably lower levels. These small polydisperse circular DNA (spcDNA) molecules (21) are found in all mammalian and avian cell types examined. For example Yamagishi et al. (29) identified spcDNA in chicken bursa, in chicken and mouse thymus, and in primary cell cultures from chicken retina and mouse liver as well as in several established cell lines such as HeLa, 3T6, and two embryonal carcinoma lines. They conclude that spcDNA is ubiquitously present and that smaller (<3-kilobase [kb]) molecules predominate in established cell lines, whereas larger molecules also are present in primary cultures and cells in situ. Cloning and molecular characterization of such molecules from mammalian cell lines have been undertaken by, among others, Krolewski et al. (13) and Stanfield and Helinski (24). They find that spcDNA is, as indicated by the designation, heterogeneous with respect to both size and sequence. All clones from spcDNA characterized so far hybridize with chromosomal DNA, with one exception (24). There are several indications that the cellular spcDNA content can vary. spcDNA has been implicated in differentiation in chicken bursa (6, 25) and in the early mouse embryo (28). Yamagishi et al. (28) argue for larger spcDNA to be specifically involved in cellular differentiation, since these larger molecules appear at a time at which the determination process is proposed to take place. The spcDNA of in vitro-cultured cells amplifies on cultivation at saturation density as well as after treatment with the protein synthesis inhibitors puromycin and cycloheximide (12, 21). Schimke (20) suggested that spcDNA is involved in gene amplification.

The present work is a more systematic study of the relationship between DNA transfection of mammalian cells and the appearance of small circular DNA in these cells a short time (42 h) after transfection.

MATERIALS AND METHODS

Cells and culture conditions. The following established cell lines were used: mouse 3T6 cells, baby hamster kidney cells (BHK 21 clone 13), and the human lymphoid cell line BJA-B. Primary embryonal mouse cells (mainly fibroblasts) were obtained from 6-day-old mouse embryos. After 5 days of in vitro culture, the cells were thoroughly washed with Mg^{2+}- and Ca^{2+}-free phosphate-buffered saline and trypsinized for use in transfection experiments. Human total peripheral lymphocytes from one healthy blood donor were prepared on Lymphoprep (Pharmacia Fine Chemicals) as described by the supplier. The lymphocytes were used the same day in transfection experiments. Cells were grown in RPMI 1640 (BJA-B and human peripheral lymphocytes) or Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

Transfection procedures. Nucleic acids were transferred by the previously described erythrocyte ghost fusion technique (27). Briefly, human erythrocytes were lysed and washed in hypotonic buffer, and DNA was added. Isotonicity was restored, and the DNA-loaded ghosts were fused with cells by using polyethylene glycol as a fusogen. Alternatively, calcium phosphate precipitation (9) with subsequent glycerol shock (19) was used. Unless stated otherwise, 10 μg of DNA was used to transfect 10^6 cells. Only exponentially growing cells were used in transfection experiments, and cells were never allowed to reach saturation density either before or after transfection. Care was taken to give control cells identical culture conditions. At every occasion when a transfection experiment was carried out, the control cells were reseeded at the same density as the transfected cells.

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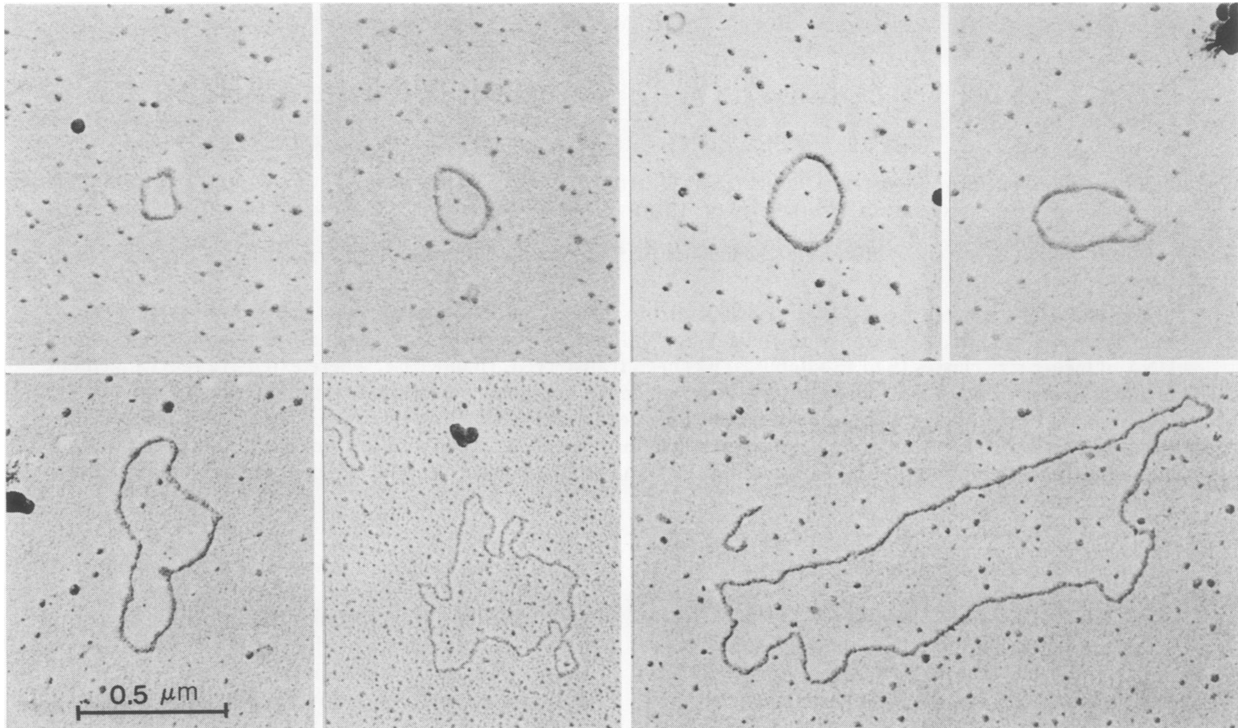

FIG. 1. Electron micrographs of circular DNA from transfected 3T6 cells. At 42 h posttransfection of pATPy, DNA was isolated and prepared for electron microscopy as described in Materials and Methods. Upper row from left to right: small circular DNA of 0.46, 0.63, 0.90, and 0.98 μm. Lower row from left to right: small circular DNA (1.35 μm), pATPy (2.73 μm), and mtDNA (4.86 μm).

Nucleic acid species used in transfection. The plasmid pAT153 has been described by Twigg and Sherrat (26). The plasmids pBR325 and pBR327 have been described by Bolivar (4) and Soberon et al. (22). Recombinant plasmid pATPy consists of the entire genome of polyomavirus strain A-2 cut with *Bam*HI and inserted into the *Bam*HI site of pAT153. Since the unique *Bam*HI site of polyomavirus is located in the late transcriptional region, this plasmid possesses an uninterrupted polyomavirus origin and early transcriptional region. Recombinant plasmid 327PyEco has the entire, *Eco*RI-cut polyomavirus genome inserted into the *Eco*RI site of pBR327. In this plasmid, the coding sequence of large T antigen of polyomavirus is interrupted by the insertion of pBR327. Hence, this plasmid is regarded as unable to synthesize large T antigen. All bacterial plasmids were prepared from *Escherichia coli* HB101 by alkaline lysis followed by equilibrium centrifugation in CsCl-ethidium bromide. *Hin*dIII-cleaved wild-type lambda DNA was obtained from Amersham Corp. Polyomavirus DNA was prepared from 3T6 cells infected at low multiplicity with polyomavirus strain A-2 by the Hirt procedure (11) and further purified by equilibrium centrifugation in CsCl-ethidium bromide and sedimentation in neutral surcrose gradients. Total *E. coli* RNA, DNA free, was purchased from Sigma Chemical Co. and further purified by precipitating twice with cetylpyridine. All DNA species used in transfections were thoroughly characterized by gel electrophoresis and Southern blotting. Some species were also examined by electron microscopy.

Preparation of Hirt supernatants from transfected and control cells. After transfection, cells were grown for 42 h, or in one case (Table 1, experiment 9) for 20 min. Before harvest, cells were rinsed three times with phosphate-buffered saline to remove excess ghosts. Cells were lysed and centrifuged, and DNA was prepared from the supernatants as described by Hirt (11).

Gel electrophoresis and Southern blotting. Electrophoresis was carried out in Tris-acetate buffer at 1.2 V/cm in 1% agarose gels. Standard methods were used for transfer to nitrocellulose filters and hybridization (23) to nick-translated probes (17). The filters were washed in 0.2× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate)–0.2% sodium dodecyl sulfate at 68°C three times for 45 min.

Electron microscopy. Portions of the Hirt supernatants were spread with an aqueous droplet technique, and contour lengths were measured as described earlier (27). Lengths were calculated relative to an internal length standard, namely, the circular mitochondrial DNA (mtDNA) originating from the recipient cells. The sizes of mtDNA are as follows: for 3T6 and primary mouse cells, 16,295 base pairs (2); for BJA-B and peripheral human lymphocytes, 16,569 base pairs (1). The exact size of hamster mtDNA is not known, but since mammalian mtDNAs are known to have very similar sizes in the range of 16 to 17 kb, a value similar to that of mouse mtDNA, 16.3 kb, is assumed for BHK cells.

RESULTS

Generation of small circular DNA is an intracellular event. To ascertain that the DNA to be transfected was homogeneous and not contaminated by small circular DNA, each DNA species was analyzed by gel electrophoresis and Southern blotting. In each case only form I (supercoiled), form II (open circular), and form III (linear) molecules of unit length could be detected (Fig. 2). Electron microscopy of the pATPy preparation revealed only circular molecules of unit length.

Next we examined whether nuclease activity in ghosts or in culture medium where cells were grown could affect the

TABLE 1. Statistical analysis of the number of small circular DNA molecules in transfected and control cells[a]

Expt no.	Cells	Treatment	Transferred DNA (except no. 18)	Size of transfected circular DNA (kb)	New bands of transferred DNA[b]	No. of circular molecules: Small circular DNA	Transfecting DNA	mt DNA	Total	Relevant control expt. no.	χ_2 compared with control[c]		Number of small circular molecules per cell[d]	Fold increase relative to control[e]	See Fig. no.
1	3T6, 4 × 10^7	GF	pATPy, 4 μg	9.0	–	18	3	37	58	19	3.2	$0.05 < P < 0.1$	490	(2.9)	2A, lane 10; 4B
2	3T6, 2 × 10^7	GF	pATPy, 40 μg	9.0	+	81	9	4	94	19	80.5	$P < 0.001$	20,000	120	2A, lane 7; 4C
3	3T6, 1 × 10^7	GF	pATPy	9.0	–	39	3	52	94	20	9.4	$0.001 < P < 0.01$	750	4.4	2A, lane 4
4	3T6, 1 × 10^7	GF	pATPy, 3 μg	9.0	–	21	1	21	43	20	10.7	$0.001 < P < 0.01$	1,000	5.9	2A, lane 11; 3C
5	3T6, 1 × 10^7	GF	pATPy, 300 μg	9.0	–	26	5	24	55	20	13.1	$P < 0.001$	1,080	6.4	2A, lane 3
6	3T6, 1 × 10^6	GF	pATPy	9.0	–	27	3	25	55	21	31.0	$P < 0.001$	1,080	9.0	2A, lane 21
7	3T6, 1 × 10^7	GF, 20 min[f]	pATPy, 3 μg	9.0	–	2	10	26	38	20	0.9	$P < 0.2$	80	(0.5)	2A, lane 14; 3B
8	3T6, 1 × 10^7	Mock GF			NA	8	NA	26	34	20	0.2	$P > 0.2$	310	(1.8)	2A, lane 13
9	3T6, 1 × 10^6	Mock GF			NA	6	NA	41	47	21	0.0	$P > 0.2$	150	(1.2)	2A, lane 23
10	3T6, 1 × 10^6	CaP	pATPy	9.0	–	28	1	36	65	21	23.0	$P < 0.001$	780	6.5	2A, lane 24
11	3T6, 1 × 10^6	CaP	pATPy	9.0	–	13	7	36	56	22	7.4	$0.001 < P < 0.01$	360	3.8	2A, lane 26
12	3T6, 1 × 10^6	Mock CaP			NA	3	NA	46	49	22	0.0	$P > 0.2$	65	(0.7)	2A, lane 28
13	3T6, 1 × 10^6	GF	Polyomavirus	5.3	–	17	5	39	61	20	2.0	$0.1 < P < 0.2$	440	(2.6)	2B, lane 2
14	3T6, 1 × 10^6	GF	pAT153	3.7	–	19	17	30	66	20	5.2	$0.01 < P < 0.05$	630	3.7	2C, lane 2; 5A
15	3T6, 1 × 10^6	GF	pBR325	6.0	–	14	3	48	65	22	5.3	$0.01 < P < 0.05$	290	3.1	2D, lane 2
16	3T6, 1 × 10^6	GF	327Py Eco	8.6	–	24	2	31	57	21	21.7	$P < 0.001$	770	6.4	2E, lane 2; 5B
17	3T6, 1 × 10^6	GF	λ *Hin*dIII	NA	–	18	NA	20	38	21	22.0	$P < 0.001$	900	7.5	2F, lane 2; 5C
18	3T6, 1 × 10^6	GF	RNA, 40 μg	NA	NA	25	NA	66	91	20	1.5	$P > 0.2$	380	(2.2)	
19	3T6, 1 × 10^7	Control			NA	6	NA	35	41				170		2A, lane 2; 4A
20	3T6, 1 × 10^7	Control			NA	10	NA	48	58				170		2A, lane 12
21	3T6, 1 × 10^6	Control			NA	12	NA	100	112				120		2A, lane 22
22	3T6, 1 × 10^6	Control			NA	9	NA	96	105				95		2A, lane 27
23	PMC, 1 × 10^6	GF	pATPy	9.0	–	60	14	13	87	24	52.0	$P < 0.001$	4,600	11.5	2A, lane 6; 6B
24	PMC, 1 × 10^6	Control			NA	24	NA	60	84				400		2A, lane 9; 6A
25	BHK, 1 × 10^6	GF	pATPy	9.0	–	26	6	68	100	26	5.4	$0.01 < P < 0.05$	380	2.4	2A, lane 19; 6D
26	BHK, 1 × 10^6	Control			NA	15	NA	95	110				160		2A, lane 20; 6C
27	BJA-B, 1 × 10^6	GF	pATPy	9.0	–	9	8	39	56	28	0.0	$P > 0.2$	230	(0.9)	2A, lane 17
28	BJA-B, 1 × 10^6	Control			NA	9	NA	36	45				250		2A, lane 18
29	HPL, 1 × 10^6	GF	pATPy	9.0	–	117	7	7	131	30	2.3	$0.1 < P < 0.2$	340	(2.4)	2A, lane 5
30	HPL, 1 × 10^6	Control			NA	71	NA	10	81				140		2A, lane 8

[a] Abbreviations: GF, ghost fusion; NA, not applicable; CaP, calcium phosphate precipitation; PMC, primary mouse cells; HPL, human peripheral lymphocytes.
[b] Bands seen in Southern blot of DNA from transfected cells that do not exist in the originally transferred DNA and that hybridize to the transferred DNA.
[c] Chi-square analysis (2 × 2) with Yates' continuity correction of the number of small circular and mtDNA molecules found in control and transfected cells.
[d] Assuming the constant number of 1,000 mtDNA molecules per cell (5), except for human peripheral lymphocytes where a value of 20 mtDNA molecules per cell is assumed (13).
[e] In the cases where no statistically significant ($P < 0.05$) increase is seen, this value is put between parentheses.
[f] pATPy was transferred into 3T6 cells as in other experiments, but DNA was isolated at 20 min posttransfection.

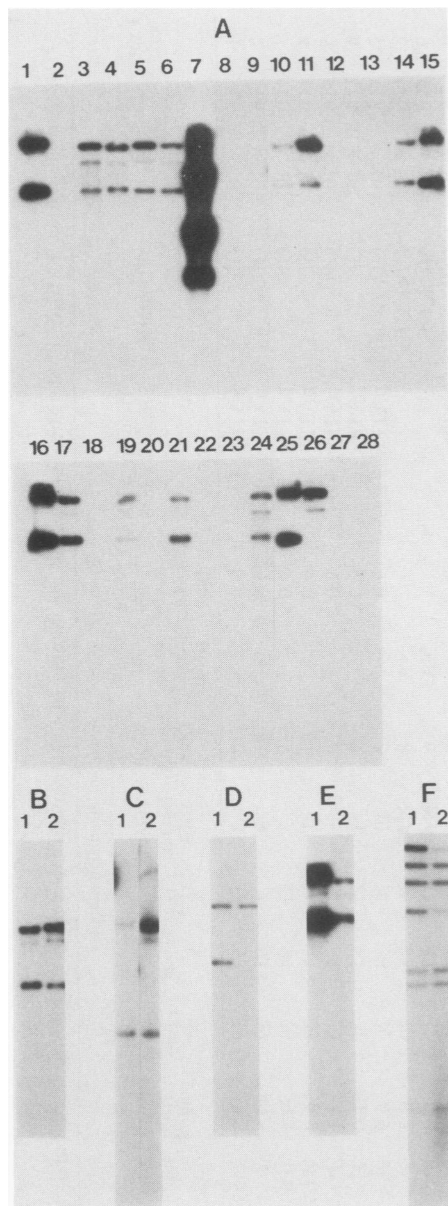

FIG. 2. Southern blot analysis of DNA extracted from transfected and control cells. Hirt supernatants were applied undigested to a 1% agarose gel. After electrophoresis and transfer to nitrocellulose filters the DNA was hybridized to the same DNA that was used in that particular transfection experiment, which was also used as a marker on the gels. A, pATPy-transfected and control cells. Lanes: 1, marker pATPy; 2, control 3T6 cells; 3, pATPy (300 μg) used to transfect 10^7 3T6 cells; 4, pATPy (10 μg) used to transfect 10^7 3T6 cells; 5, peripheral human lymphocytes transfected with pATPy; 6, primary mouse cells transfected with pATPy; 7, pATPy (40 μg) used to transfect 2×10^7 3T6 cells; 8, control human peripheral lymphocytes; 9, control primary mouse cells; 10, pATPy (4 μg) used to transfect 4×10^7 3T6 cells; 11, pATPy (3 μg) used to transfect 10^7 3T6 cells; 12, control 3T6 cells; 13, 3T6 cells subjected to mock ghost fusion; 14, pATPy (3 μg) used to transfect 10^7 3T6 cells and DNA isolated 20 min posttransfection; 15, pATPy reextracted from loaded ghosts; 16, marker pATPy; 17, BJA-B cells transfected with pATPy; 18, control BJA-B cells; 19, BHK cells transfected with pATPy; 20, control BHK cells; 21, 3T6 cells transfected with pATPy; 22, control 3T6 cells; 23, 3T6 cells subjected to mock ghost fusion; 24, 3T6 cells transfected with pATPy with calcium phosphate precipitation; 25, marker pATPy; 26, 3T6 cells transfected with pATPy with calcium phosphate precipitation; 27, control 3T6 cells; 28, 3T6 cells subjected to mock calcium phosphate precipitation. B through F, Different DNA species used to transfect 3T6 cells. In each case lane 1 represents the marker and lane 2 shows DNA extracted from transfected cells. In C and F the gel has been run longer than in the rest of the lanes. The following DNA species were transferred: B, polyomavirus; C, pAT 153; D, pBR 325; E, 327PyEco; F, *Hin*dIII-digested lambda DNA.

loaded DNA. For this purpose, DNA loaded in ghosts was reextracted and analyzed either directly or after coincubation at 37°C in culture medium with 3T6 cells. Again, no DNA molecules other than unit length were found either by electron microscopy (Fig. 3A) or Southern blotting (Fig. 2A, lane 15). It is concluded from these studies that the transfecting DNA is not in any way altered to form small DNA circles or any other rearrangement products by the handling of DNA and ghosts up to the point of entry into cells (i.e., the polyethylene glycol-induced fusion).

It might be argued that smaller circular DNA molecules, too few to be detected by electron microscopy or Southern blotting, could preferentially enter cells to produce the size distribution of circular DNA observed in transfected cells. To investigate this possibility we isolated DNA from transfected cells a short time (20 min) after transfer (Table 1, experiment 7; Fig. 3B). No increase in the number of small circles relative to mtDNA was seen. Hence, the expansion of the pool of small circular DNA must take place between 20 min and 42 h posttransfection (Fig. 3C).

Formation of small circular DNA is not caused by polyethylene glycol-induced fusion or calcium phosphate precipitation. As reviewed in the introduction, the amount of spcDNA is known to be influenced by a number of external factors, including cell density. It could therefore seem conceivable that the altered conditions and stress imposed on cells during erythrocyte ghost fusion-mediated transfection could be responsible for this effect, even though control cells were cultured identically and at the same densities as transfected cells before and after transfection. This possibility was ruled out by mock fusions where empty ghosts were fused with 3T6 cells, where no increase of spcDNA was seen (Table 1, experiments 8 and 9). Another point we wanted to clarify was whether the observed effect is specific for the mode of DNA entry into cells inherent to the ghost fusion technique. To this end the same amount and type of DNA (pATPy) was used to transfect the same number and type of cells (3T6) by calcium phosphate precipitation with subsequent glycerol shock. Virtually identical results were obtained as with ghost fusion, namely, an about fivefold increase of small circular DNA (Table 1, experiments 10 and 11). Also, a mock $CaCl_2$ precipitation without added DNA was carried out (Table 1, experiment 12). Just as with mock ghost fusion, no effect was seen.

Southern blot analysis of transfected DNA. The Hirt supernatants isolated from transfected cells at 42 h posttransfection were analyzed undigested by Southern blotting. When two clones of repetitive mouse sequences were used as a probe (where mouse cells were used as recipients), no extrachromosomal hybridization could be detected (data not shown). When instead the transfecting DNA was used as a probe (Fig. 2), the rule was that the transfected DNA appeared unchanged by transfection, except for occasional form I-to-form II conversion when compared with the DNA that was actually transfected. Even at prolonged exposure, no smear or bands of degraded DNA appeared. This applied to all combinations of recipient cells and transfecting DNA

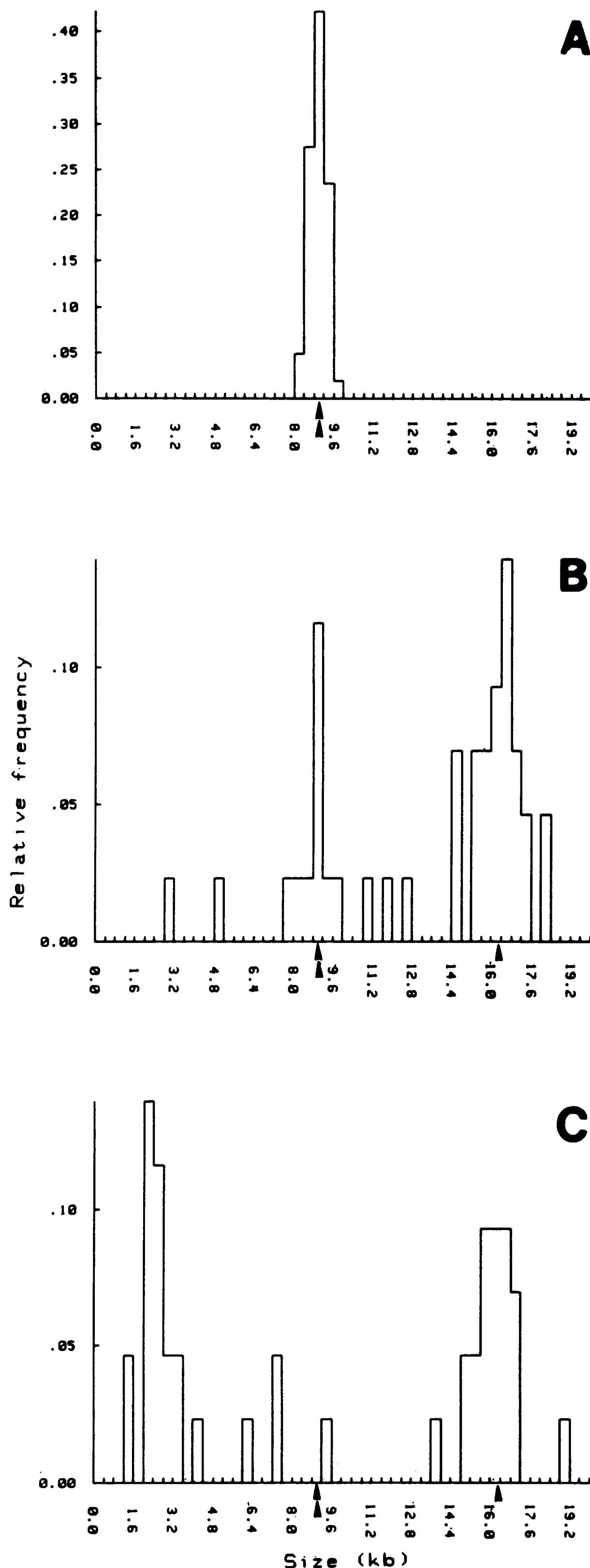

FIG. 3. Size distribution of circular DNA before, immediately after, and 42 h after transfection of 10^7 3T6 cells with 3 μg of pATPy. DNA was prepared for electron microscopy, circular molecules were photographed, and their contour lengths were digitized as described in Materials and Methods. The size of mtDNA of 3T6 cells, 16,295 base pairs, is indicated with an arrowhead in the histogram. The size of the transfected pATPy, 9.0 kb, is marked by double arrowheads. A, pATPy reextracted directly from loaded ghosts; B, cells harvested 20 min after transfection; C, cells harvested 42 h after transfection.

except *Hin*dIII-cleaved lambda. The selective degradation shown in Fig. 2F, lane 2, of lambda *Hin*dIII fragments 1 and 4, in which the single-stranded *cos* sequences are located, was consistently observed after introduction into mammalian cells (our own observations). Part or all of this degraded mass is seen as a smear with a maximum around 0.5 kb (lambda *Hin*dIII fragment 7). This is in contrast to experiments where other DNAs are introduced. There is one other notable exception. Out of the six transfections with pATPy and 3T6 (Table 1, experiments 1 through 6), experiment 2 showed a multitude of bands migrating faster than form I pATPy (Fig. 2A, lane 7). By probing the blot first with pAT alone and then with the entire pATPy, we found that almost all new bands were positive for polyomavirus but not for pAT. A similar analysis after cleavage with various restriction enzymes confirmed this finding.

Molecular cloning of rearranged pATPy derivatives. The DNA from experiment 2 was cleaved with *Bam*HI and cloned in pBR322. Of the polyomavirus-positive clones, 75% had an insert of similar size as wild-type polyomavirus. The remaining 25% fell into two categories with an insert of 2.0 or 2.3 kb, respectively. Both these classes of clones were positive for polyomavirus *Hpa*II fragments 1, 3, and 5 (data not shown). In an alternative cloning procedure the same material was cleaved with *Hae*II, dC tailed, and annealed to *Pst*I-cleaved, dG-tailed pUC9. Of the polyomavirus-positive clones isolated, 80% had an insert of a size between 1.1 and 1.3 kb. All of these clones that were tested turned out to be positive for polyomavirus *Hpa*II fragments 3, 4, and 5. The remaining 20% of the clones had an insert corresponding to full-length polyomavirus.

Thus, all polyomavirus recombinant clones isolated have retained parts of polyoma *Hpa*II fragments 3 and 5, where the polyomavirus origin of replication is located.

Electron microscopic studies. In histograms showing the size distribution of circular DNA in transfected cells at 42 h posttransfection, the transfected DNA did not make out a prominent peak (Fig. 3C, 4 through 6). mtDNA was seen as a homogeneous peak at 16 kb. The most apparent difference between control and transfected cells was the elevated level of small circular DNA, which was found as a continuum from 1 kb up to about 7 kb.

The variation inherent in electron microscopy of nucleic acids makes a population of identical double-stranded DNA molecules appear in a histogram of measured contour lengths as a Gaussian curve. This variation and the fact that the sizes of transfected DNA and spcDNA in some cases overlap pose problems which have to be considered when assigning an individual molecule to one of the size classes small circular DNA (A), transfected DNA (B), or mtDNA (C). This was addressed as follows.

(i) The width of classes B and C was set at mean ± 3 standard deviations, where one standard deviation equals k multiplied by the square root of the molecule length (5). In our analyses with the aqueous technique we have determined k to be 0.1 when the molecule length is expressed in kilobases.

(ii) In untransfected cells, all circular molecules falling below the lower limit of class C are recorded as "small

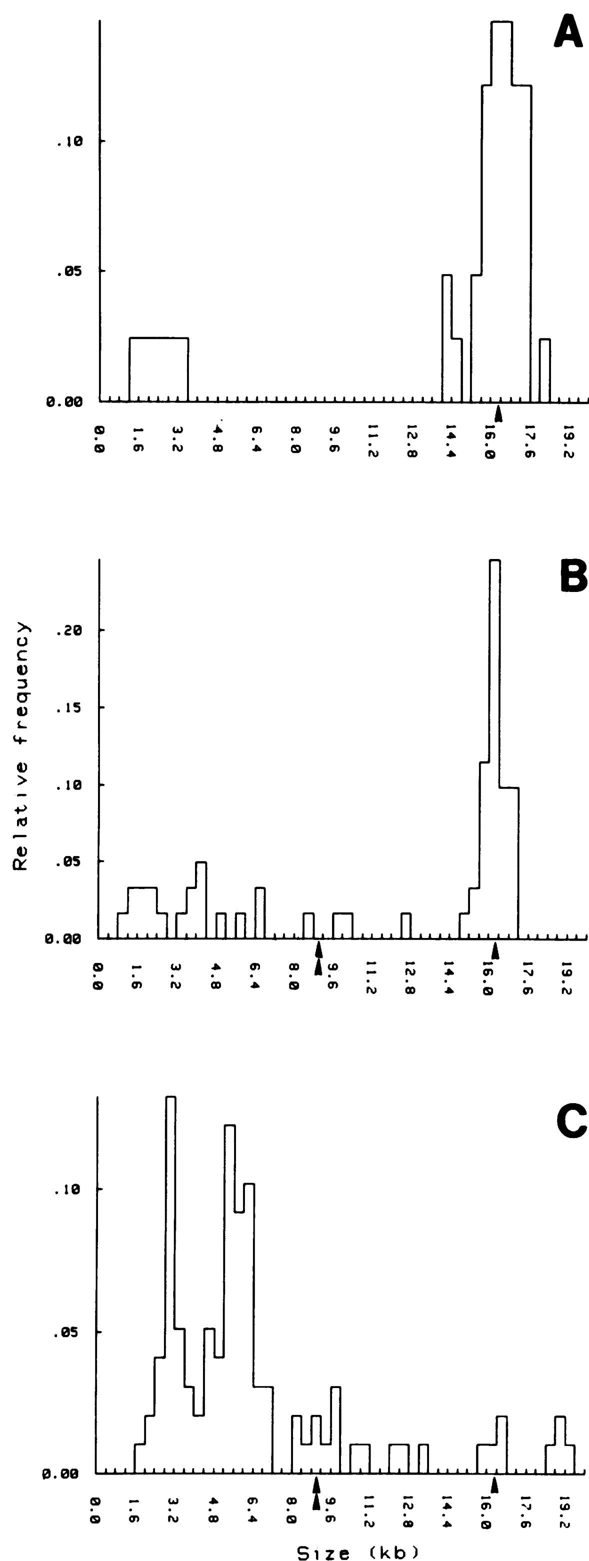

FIG. 4. Length distribution of circular DNA in Hirt supernatants from control and transfected 3T6 cells at 42 h posttransfection. Procedure and designations were as in Fig. 3. A, Control cells; B, 4 μg of pATPy used to transfect 4×10^7 cells; C, 40 μg of pATPy used to transfect 2×10^7 cells.

circular DNA," but in cells transfected with circular DNA, only molecules falling below class B are assigned to small circular DNA.

To quantitate the number of small circular DNA molecules, mtDNA was used as a number standard as well as a size standard. A constant number of 1,000 mitochondrial genomes per cell is assumed (3), except for peripheral lymphocytes where the number is considerably lower, some 20 per cell (10). That the amount of mtDNA is indeed constant was established by Southern blotting experiments where equal amounts of total Hirt supernatant DNA from transfected and control mouse cells (primary and 3T6 cells) were hybridized to the purified inserts of cloned mouse mtDNA *Bam*HI fragments 1 through 4 (G. Bjursell, unpublished data). No change in hybridization intensity was observed (data not shown). Also, the ratio of mtDNA to linear DNA appeared unchanged, whereas the number of small circular DNAs per electron microscopic grid was increased in transfected cells.

Under these assumptions, the number of transfected circular molecules was calculated to be typically in the range of 30 to 300 per cell, averaged over the whole cell population (Table 1). Assuming that 20% of the cells take up DNA (27), this number would be 150 to 1,500 per cell. Analogously the small circular DNA amounted to about 150 molecules per cell in the control population and 500 to 1,000 molecules per cell in the transfected population, which corresponds to a fivefold increase upon transfection. If the circular DNA content in cells that do not actually receive DNA is unaltered, as indicated by mock transfections, then the small circular DNA in the 20% that do take up DNA is increased to 2,500 to 5,000 molecules per cell, which corresponds to a 25-fold expansion. The number ratio of small circular DNA to transfected DNA was in most cases between 5 to 1 and 15 to 1. Given a mean size of 2.0 kb of small circular DNA, this means in cases where pATPy (9.0 kb) was transferred that the mass of small circular DNA was about twice that of transfected DNA.

Increase of small circular DNA is nonspecific with respect to type of transferred DNA. So far all our studies were performed with pATPy introduced into 3T6 cells. To find out whether the observed effect was specific for any particular transfecting DNA, we conducted a series of experiments where different DNA species were transfected into 3T6 cells.

When polyomavirus DNA (5.3 kb) or pAT (3.7 kb) was introduced, an increase in small circular DNA was seen at levels comparable with those when pATPy was used (Table 1, experiments 13 and 14). However, the picture was complicated by the small sizes of these transfected DNAs, which in fact are within the range of spcDNA (Fig. 5A). These complications were partially obviated when using the slightly larger plasmid pBR325 (6.0 kb). The same type and level of increase was again seen (Table 1, experiment 15). Plasmid 327PyEco has a size (8.6 kb) similar to that of pATPy but cannot synthesize intact large T antigen. When this plasmid was introduced, small circular DNA was produced in the same amount (Table 1, experiment 16; Fig. 5B) as when using pATPy. A role of large T antigen in this process thus seems very unlikely. This was further confirmed when it was found that a completely unrelated DNA species, wild-type phage lambda DNA digested with *Hin*dIII, was able to produce the same effect (Table 1, experiment 17; Fig. 5C). From this experiment it can also be learned that it is not a prerequisite for the generation of small circular DNA that the introduced DNA be circular.

We wanted to see whether the observed effect could be

seen with other nucleic acids. Hence total *E. coli* RNA (40 μg) was loaded in ghosts which were fused with 3T6 cells in the same way as when transferring DNA. No statistically discernible increase of small circular DNA was observed (Table 1, experiment 18).

Different cell types differ in their response to incoming DNA. Having established that several different DNA species are seemingly equally capable of provoking the described response in 3T6 cells, we turned to other recipient cell types, again using pATPy as the transfecting DNA. First the size distributions of circular DNA in normal, untransfected cells were examined. Comparable amounts of spcDNA as in 3T6 cells relative to mtDNA were found in BHK, BJA-B, and primary mouse cells (Table 1, experiments 24, 26, and 28). The size distributions of spcDNA in these cells were also similar to that of 3T6 cells (Fig. 6A and C). In human peripheral lymphocytes the number of mtDNA molecules per cell is much lower (10), and consequently mtDNA makes out only a minor fraction of the circular DNA, the majority being spcDNA (Table 1, experiment 30). In primary mouse cells the increase in small circular DNA was the greatest, about 12-fold (Table 1, experiment 23; Fig. 6B). Also, in these cells the mean size of the small circular DNA (size class A) increased from 2.0 to 2.9 kb upon transfection. In BHK cells the increase, 2.5-fold (Table 1, experiment 25; Fig. 6D) was less pronounced than that typically seen in 3T6 cells. BJA-B cells did not display any effect upon transfection (Table 1, experiment 27). In human peripheral lymphocytes no statistically significant change could be observed due to the low amount of mtDNA (Table 1, experiment 29).

DISCUSSION

Source of new small circular DNA. One major question which has only been briefly touched upon so far is the origin of the new small circular DNA. There are two possibilities: they are derived either from the transfecting DNA or from the DNA of the recipient cells.

If extensive recombination of the transferred material has taken place in experiment 2, there is no sign of such recombination in the other experiments where DNA has been transfected. However, there is ample evidence that transfected DNA is subject to an increased level of different types of mutational and recombinational events in mammalian cells (7, 15, 16). These workers have specially designed systems in which to select for mutated molecules. In contrast, we have directly analyzed the total population of transfected molecules by Southern blotting. Mutations including rearrangements at a frequency comparable to that estimated by these authors, in around 1% of transferred molecules, may well be present in the other experiments where DNA is transferred. However, this cannot account for the about fivefold increase of small circular DNA that consistently occurs in transfection experiments. No material of this size (1 to 5 kb) hybridizing to the transfected DNA is detected on Southern blots (except in experiment 2). As calculated in Results, the mass of the new small circular DNA is on the average twice that of the transfected DNA. It is estimated that about 1 ng of transfecting DNA in each experiment has been applied to the gel. If it is assumed that all of the newly produced small circular DNA is derived from the transfecting DNA, then 2 ng of probe-reactive material should be present in the 1- to 5-kb region, with a maximum at about 2 to 3 kb. If these values are corrected for the preexisting spcDNA, they drop only 20%. Given the high sensitivity of Southern blotting where 1 pg in one band can be detected, an amount of DNA of several thousandfold this

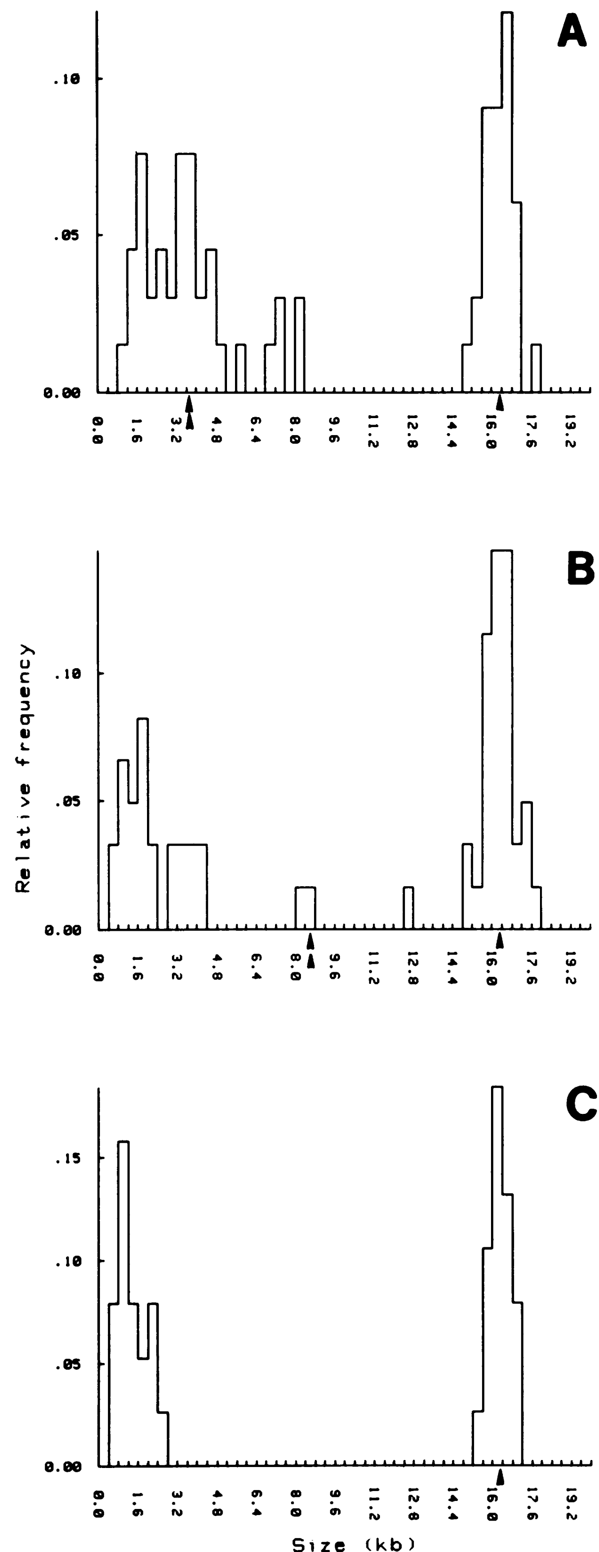

FIG. 5. Size distribution of circular molecules in 3T6 cells at 42 h after transfection of 10 μg of different DNA species with 10^6 cells. Procedure and designations were as in Fig. 3. The following DNAs were transferred: A, pAT (3.7 kb); B, 327PyEco (8.6 kb); C, *Hin*dIII-digested lambda DNA. This DNA is not circular, so there are no double arrowheads to mark its size.

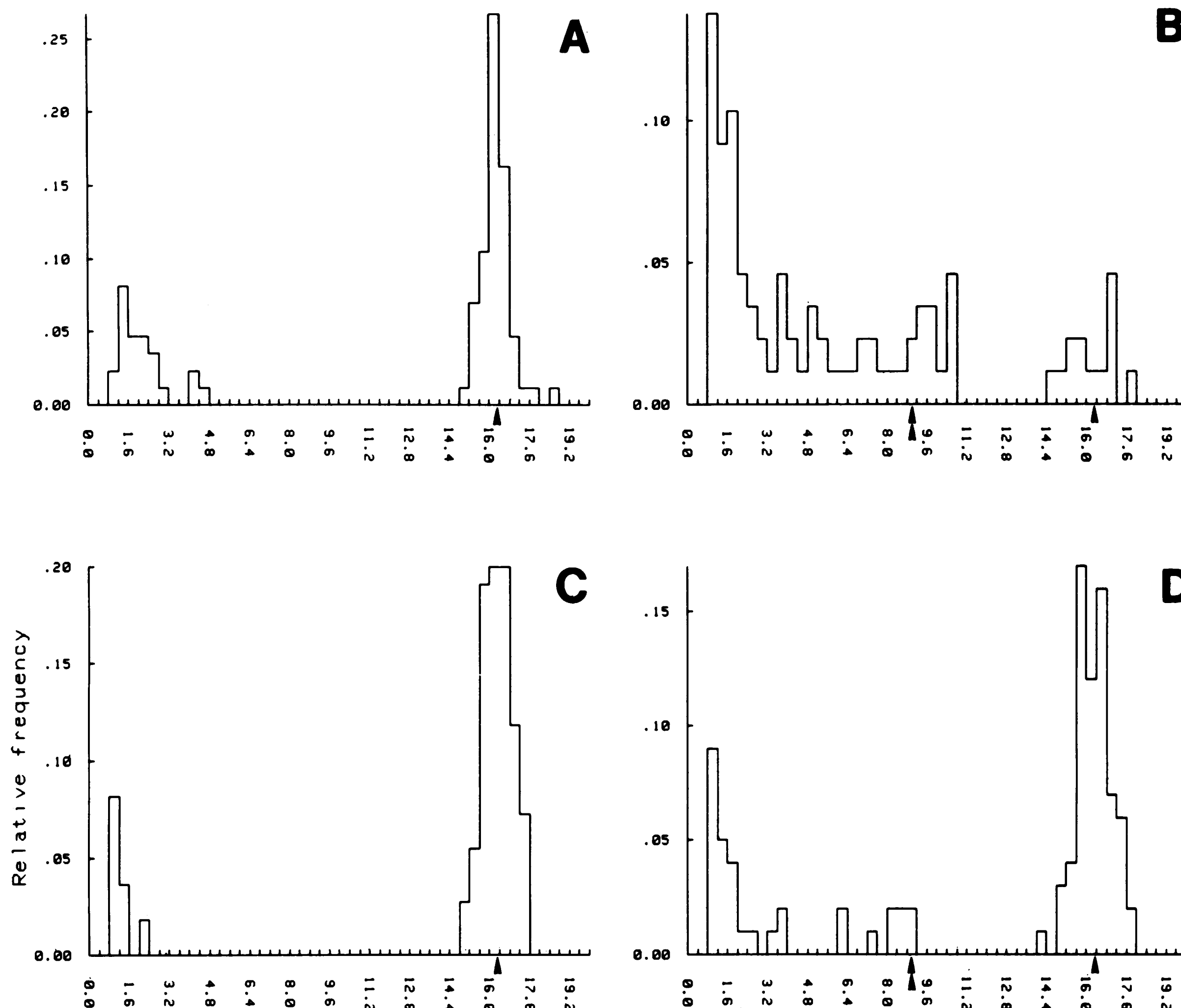

FIG. 6. Size distribution of circular DNA at 42 h after transfection in different cell types with or without transfection of pATPy. A 10-μg sample was used to transfect 10^6 cells. Procedure and designations were as in Fig. 3. A, Control primary mouse cells (mtDNA, 16,295 base pairs); B, primary mouse cells after transfection with pATPy; C, control BHK cells (mtDNA, 16.3 kb); D, BHK cells after transfection with pATPy.

value should be quite intensely labeled if hybridized with a probe homologous to all of this material. But not even at prolonged exposure, where the bands of transfected DNA are strong, was such a hybridization seen.

This fact makes it logical to consider the alternative explanation, that the major fraction of the new small circular DNA is derived from the cellular DNA.

With the knowledge that spcDNA can be induced to increase by such a variety of factors, it is not implausible that a similar amplification could take place in response to incoming DNA. We have not been able, however, to positively verify such an interpretation by hybridizing with mouse DNA probes. When evaluating this lack of result of that very preliminary investigation, it is necessary to keep the following point in mind. All reports on the molecular composition of spcDNA agree on its sequence heterogeneity. If the small circular DNA produced in transfected cells is interpreted as related to spcDNA, even the most highly repeated sequence may recognize only a small fraction of the small circles.

We therefore think that the most likely interpretation is that we observe the combined effect of two mechanisms: (i) rearrangement of transfected DNA and (ii) induction of small cellular DNA circles, where the relative contributions of these mechanisms vary from experiment to experiment. Experiment 2 would then represent one extreme where a significant proportion clearly is due to rearrangement of transfected DNA. In the remaining experiments, where it is difficult to see how such rearrangement could account for the large amount of small circles produced, amplification of the pool of cellular spcDNA would be the predominant mechanism.

Which factors are essential for the formation of small circular DNA? It has been shown by assaying transient expression and cell transformation that DNA introduced by the modified erythrocyte ghost fusion technique does in fact reach the nucleus and becomes expressed, both in rodent fibroblastoid cells (27) and in lymphoid cells (Wiberg et al., manuscript in preparation). Calcium phosphate precipitation is also a well-documented method of introducing DNA into cells. Since no changes can be detected in the transfecting DNA either before or indeed (with the two exceptions discussed above) at 42 h after transfection, the small circular DNA must be produced intracellularly.

No difference with respect to the extent of the effect was seen between the two transfection methods used; as discussed above, neither mock transfection treatment without added DNA gave any effect. If any steps in the way DNA enters cells in ghost fusion-mediated transfer are responsible for the generation of small circular DNA, then these steps are shared by the more widely used calcium phosphate precipitation technique.

Likewise, no significant differences are seen between the tested DNA species. It can be estimated that the degradation of lambda *Hin*dIII fragments 1 and 4 discussed in Results probably cannot account for the amount of small circular DNA seen in the corresponding histogram (Fig. 5C). The effect seems to be a general response to incoming DNA, since any specific sequences that would signal production of small circular DNA would have to be very short to occur by chance in such quite unrelated sequences as pBR derivatives, polyomavirus, and phage lambda. We have not found introduction of RNA to lead to a statistically significant production of small DNA circles. This could reflect a true difference between DNA and RNA, but considerable caution is necessary when drawing conclusions in this case. The RNA could be degraded during loading, or, once inside the cells, it may not be transported to the nucleus. More refined experiments are required to settle this issue.

Different degrees of response were seen among the cell types tested. Since the strongest response occurred in 3T6 and primary mouse cells, which are permissive for polyomavirus replication, and since the polyomavirus semipermissive BHK cells respond more weakly when pATPy is introduced, it might be held that permissiveness for replication is coupled with generation of small DNA circles. Probably this interpretation has to be abandoned, since purely bacterial plasmids and phage lambda, which are quite incapable of replication in mammalian cells, give the same effect in 3T6 cells. Rather, the observed differences probably reflect more fundamental properties of the recipient cells. In this context it is interesting to note that more larger circles are produced in primary mouse cells. This may relate to the observation that primary cells and intact tissues contain more large spcDNA molecules than established cell lines (29).

As far as we have been able to show, the production of small DNA circles seems to be a general response to any DNA coming into the cell. It can be speculated that this situation mimics a virus infection where nucleic acid enters through the cytoplasm. Since this is the route of DNA entry both in calcium phosphate precipitation and erythrocyte ghost fusion, a different result might be obtained when DNA is introduced directly into the nucleus, e.g., by microinjection. Production of spcDNA has been interpreted to result from chromosomal rearrangements which take place, for example, in differentiation of lymphocytes (8, 25). When foreign DNA is introduced into mammalian cells, as a rule it is known to finally integrate into host chromosomes after a complex series of recombinational events that also involve host sequences (18). The appearing small circular DNA we observe after transfection could be the precursors or the products of such integrational events. There have been reports on integration of cellular DNA into transfected and rescued DNA molecules (15, 16) as well as into the virus genome in a regular infection (14). The (possibly induced) spcDNA could be the source of such cellular sequences.

Considerations for further work. We have described a biological phenomenon of some generality (for several recipient cells and transfecting DNA species) which has not to our knowledge been reported before. Since DNA transfection is a commonly used technique, this effect is of general interest to the many workers employing this approach. We have only relatively coarsely delineated this phenomenon. However, it is not feasible to make a more detailed description with electron microscopy as the main tool, since it is extremely time consuming to analyze in this manner preparations where only a few percent of the molecules are circular. Experiments where a greater number of variables are investigated will have to be performed with some kind of DNA hybridization. The probes for such hybridizations are not available at the moment since, as discussed above, the transfecting DNA does not, in the majority of cases, recognize to any greater extent the produced small circular DNA when used as a probe. The low amount of small circular DNA in combination with its size and sequence heterogeneity means that the use of total cellular DNA as a probe does not give the required sensitivity. The first step to continue these studies is then to obtain clones of cellular DNA sequences which are sufficiently abundant in the induced small circular DNA to efficiently recognize it in filter hybridization. The choice of source DNA for such cloning is not trivial, since the sequence composition of the putatively expanded spcDNA pool may be quite different from the normal one.

In summary, we think that elucidation of the phenomenon described in this paper will help to clarify some points concerning the fate of transfected DNA as well as recombination and amplification of cellular genes.

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