JOURNAL OF VIROLOGY, Aug. 1986, p. 490–493
0022-538X/86/080490-04$02.00/0

Vol. 59, No. 2

Highly Infectious Plasmids Carrying Poliovirus cDNA Are Capable of Replication in Transfected Simian Cells

KATHERINE M. KEAN,* CZESLAW WYCHOWSKI, HELENA KOPECKA, AND MARC GIRARD

Unité de Virologie Moléculaire, Institut Pasteur, 75724 Paris Cedex 15, France

Received 28 March 1986/Accepted 25 April 1986

We examined events leading to production of infectious poliovirus upon transfection of simian cells with plasmids carrying poliovirus cDNA and simian virus 40 transcription and replication signals. The nature of the simian virus 40 promoter upstream from the poliovirus cDNA had no influence on its infectivity. A high specific infectivity was correlated with plasmid replication, dependent on expression of T antigen either encoded by the plasmid or present in host cells (COS-1).

The poliovirus genome is a single-stranded RNA molecule of positive polarity, 3′ polyadenylated (26) and 5′ linked to a small polypeptide, VPg (17). Studies of viral gene functions have been hampered by the lack of well-defined mutants. A new approach became feasible when Racaniello and Baltimore (20) showed that plasmids carrying the complete cDNA copy of poliovirus type 1 RNA were infectious upon transfection of cultured primate cells, since the virus produced is identical to that used for the synthesis of the cDNA (20, 22). Poliovirus production after transfection with cDNA is very inefficient (ca. 10 PFU/μg of DNA) compared with that after transfection with viral RNA (10^6 PFU/μg of RNA) (14), and its mechanism remains unknown. Insertion of the simian virus 40 (SV40) replication origin and early promoter into the plasmids significantly increased the production of infectious poliovirus (22; V. Racaniello, personal communication), but the infectivity of these plasmids reached a high level (>400 PFU/μg of DNA) only after transfection of COS-1 cells (8). In an attempt to clarify the mechanism of infectious poliovirus production from cDNA, we report here a comparison of the effects of different promoters on the expression of poliovirus cDNA independent of, or in conjunction with, plasmid replication.

We constructed two plasmids in which the full-length poliovirus type 1 cDNA was immediately preceded by the SV40 late promoter (Fig. 1). Plasmid pCW2-SV40 (25) was used as the source of SV40 DNA for the construction of recombinant vectors (Fig. 1A). Conversion of the *Hpa*II site at position 346 of SV40 DNA (24) to an *Xho*I site conserves intact the SV40 late promoter (11) and the major initiation sites of late transcription (4, 9). The SV40 *Bam*HI-*Xho*I (position 2533 to 346) fragment, used in the construction of pKK2, contains the SV40 replication origin (2), the promoters for both early and late transcription, the 72-base-pair repeat sequences known to act as an enhancer of transcription (1), and the sequences coding for T antigen (5). A large portion of the sequences coding for T antigen were eliminated from pKK2 by deletion of the *Bam*HI-*Nde*I (position 2533 to 4826) fragment, generating pKK8. Plasmid pSV-polio (Fig. 1B) was used as the source of poliovirus cDNA. This plasmid contains a full-length poliovirus type 1 cDNA immediately downstream from the SV40 early promoter and replication origin and upstream from the SV40 small t antigen signals for RNA splicing, polyadenylation, and termination (V. Racaniello, personal communication). The insertion of these sequences into pKK2 and pKK8 yielded pKK17 and pKK25, respectively (Fig. 1B). Thus, pKK17 carries the poliovirus cDNA together with all of the elements necessary for autonomous plasmid replication in simian cells (3, 23), whereas pKK25, like pSV-polio, cannot replicate in the absence of exogenous T antigen. Because the SV40 maturation signals are duplicated in pKK17, we verified by fine restriction mapping that no rearrangements via homologous recombination had occurred.

The biological activities of pKK17, pKK25, and pSV-polio were compared by measuring PFU production after transfection (10) of Vero and COS-1 cells (Table 1). The specific infectivity of a given cDNA was found to vary as a function of plasmid concentration, showing an optimum level which was different for each plasmid (Table 1) and then dropping sharply as the concentration was increased above this value (data not shown). The specific infectivities observed were independent of the transfection method used (calcium phosphate or DEAE-dextran).

Hartzell et al. (12) have shown that the strength of the SV40 late promoter is negligible in the absence of T antigen and DNA replication, whereas the early promoter functions efficiently. Transfection of Vero cells with pSV-polio or pKK25 allowed us to compare the effect of the SV40 early and late promoters, respectively, on the specific infectivity of poliovirus cDNA in the absence of plasmid DNA replication. Our results show that promoter strength had little or no effect on PFU production from poliovirus cDNA (Table 1). However, the infectivities observed were significantly higher than those observed in the absence of eucaryotic transcription signals (20) (data not shown). This was probably due to the presence of the SV40 enhancer because a significant increase in poliovirus production was observed even when the SV40 early promoter region was placed 2,700 base pairs away from the 5′ end of poliovirus cDNA (22).

The participation of T antigen in the infectivity of the poliovirus cDNA was indicated by the fact that only pKK17 showed a high specific infectivity in Vero cells, whereas all three plasmids were highly infectious in COS-1 cells, which constitutively express the SV40 T antigen (8). We verified by immunofluorescence staining (25) with hamster anti-SV40 T antigen immune serum that the SV40 T antigen was expressed in Vero cells transfected by pKK17. T antigen expression was detected in approximately 10 of 10^5 cells under the same conditions in which we observed 20 PFU per 10^6 cells. Thus, it seems that five times more cells expressed

* Corresponding author.

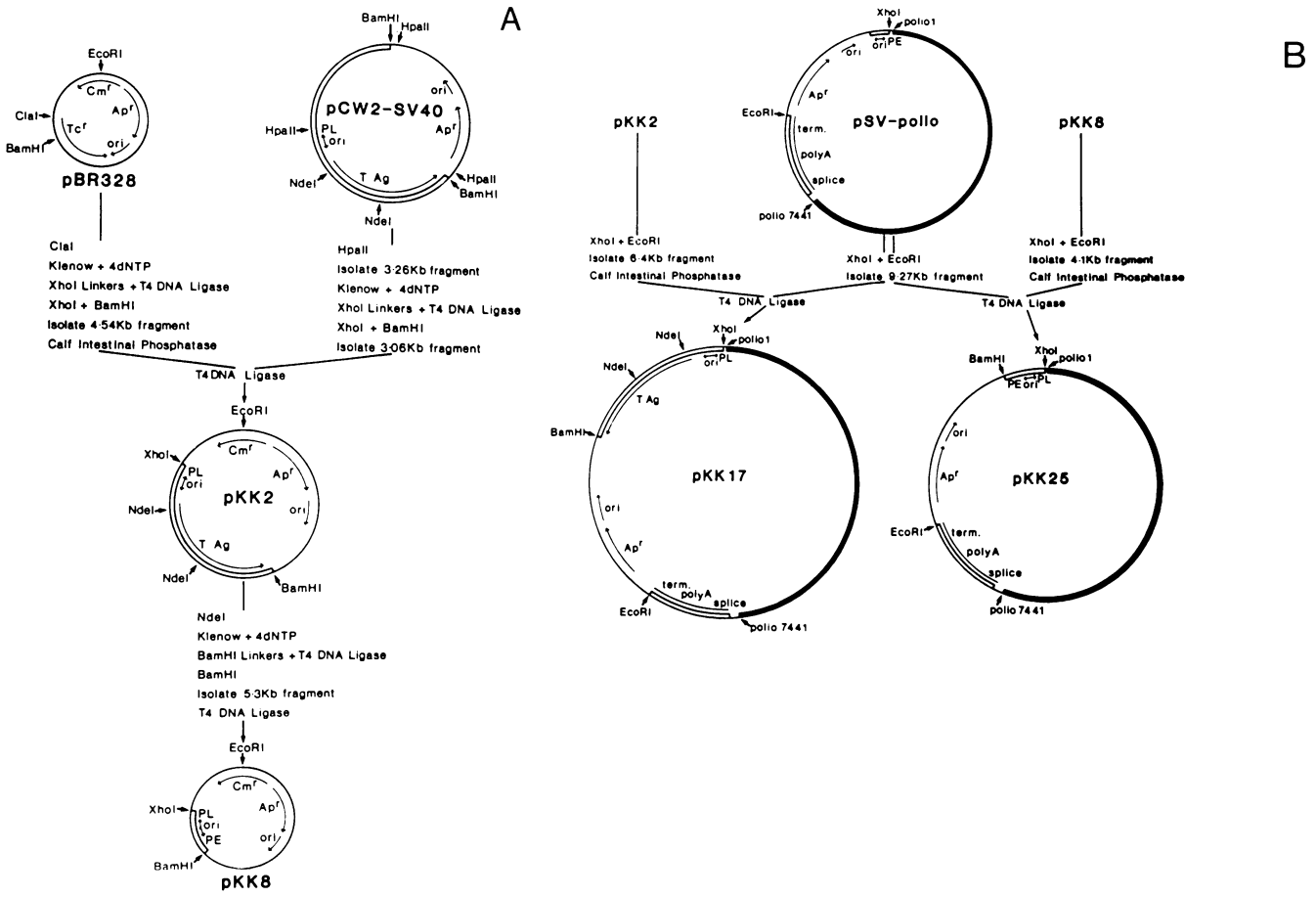

FIG. 1. Strategy for the construction of recombinant plasmids carrying a full-length infectious poliovirus cDNA. DNA was manipulated by classical techniques (18). DNA sequences derived from pBR322 and pBR328 are shown as single lines, those derived from SV40 are shown as open boxes, and those derived from the cDNA copy of poliovirus type 1 RNA are shown as closed boxes. Abbreviations: PL, late promoter; PE, early promoter; ori, DNA replication origin; splice, poly A, and term., SV40 small t antigen splice, polyadenylation, and termination signals, respectively. (A) Construction of recombinant vectors. (B) Insertion of full-length poliovirus cDNA and SV40 maturation signals.

T antigen than initiated a poliovirus PFU, although these numbers are too small to be significant.

T antigen could be involved through initiation of plasmid replication at the SV40 replication origin (3, 23) to increase the number of templates available for transcription. To measure relative plasmid copy number in simian cells at various times after transfection, biologically active plasmid molecules were scored as CFU on the basis of their capacity to transform *Escherichia coli* to ampicillin resistance. We chose this approach, rather than a simple detection of total plasmid DNA present in extracts from transfected cells, because of recent reports that transfected DNA is mutated at high frequency in animal cells (16, 21). We reasoned that, because mutations should be random, an increase in the number of plasmids with both a functional bacterial replication origin and an intact ampicillin resistance gene would be indicative of amplification of poliovirus cDNA. After transfection of Vero cells with 50 ng of DNA, only plasmids pKK2 and pKK17, which contain all of the signals for autonomous replication in simian cells, showed an increase in copy number (Fig. 2A). No increase in copy number was detected when the same quantity of DNA was used for transfection of Vero cells with pKK25 and pSV-polio (Fig. 2A), but under these conditions neither plasmid gave rise to PFU (Table 1 and data not shown). Under conditions of optimal specific infectivity of pKK25 and pSV-polio (i.e., 1.5 and 1 μg of DNA per 10^6 cells, respectively), CFU were detected, in continually decreasing amounts, from greater

TABLE 1. Specific infectivity of poliovirus cDNA in cultured simian cells

Source of DNA	Optimal conditions used (ng of plasmid/10^6 cells) for:		Virus yield (PFU/ pmol of plasmid DNA per 10^6 cells) under optimal conditions of infectivity from[a]:	
	Vero	COS-1	Vero	COS-1
pKK17	50	50	4,395	4,395
pKK25	1,500	50	53	12,700
pSV-polio	1,000	50	86	4,480
pKK2	NA[b]	NA	0	0
pKK8	NA	NA	0	0

[a] Average of five experiments. DNA was at a final concentration of 10 to 30 μg/ml, with salmon sperm DNA as carrier. Cells were seeded 24 h before transfection (10^6 cells per 60-mm plate) so that transfections were carried out on 90 to 100% confluent monolayers. After 5 h of incubation with calcium phosphate-DNA precipitates, the cells were shocked with 20% glycerol for 2 min (6).

[b] NA, Not applicable; transfection with 1.5 μg of DNA.

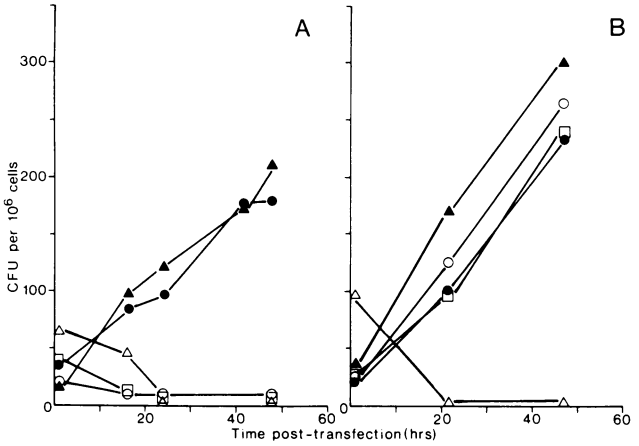

FIG. 2. Relative number of biologically active plasmid molecules present in simian cells at various times posttransfection. Vero cells (A) or COS-1 cells (B) were transfected with 50 ng of the following DNA: pKK17 (●), pKK25 (○), pSV-polio (□), pKK2 (▲), or pBR328 (△). At the indicated times, low-molecular-weight DNA was extracted from transfected cells (13), treated with RNase A (20 μg/ml for 15 min at 37°C), extracted with phenol-chloroform, and ethanol precipitated before being used to transform *E. coli* 1106 (19). Amp^r colonies were selected and scored. pBR322 DNA added to a Hirt extract from mock-transfected cells was treated in parallel and used to standardize the number of CFU. 10^6 transformants per μg of pBR322 DNA was used as a standard. Zero time was the time of glycerol shock.

than 10,000 CFU at 30 min posttransfection to 100 to 200 CFU at 48 h posttransfection (Table 2 and data not shown). In contrast, an increase in copy number was observed in COS-1 cells for all plasmids containing the SV40 replication origin (Fig. 2B). Later posttransfection, we observed a drop in copy number of replicating plasmids which we are not able to explain at present and which we did not observe with pCW2-SV40 (data not shown).

To confirm that the increases in CFU detected in the experiments shown in Fig. 2 were indeed due to plasmid replication in simian cells, DNA extracted from transfected cells was digested with one of the isoschizomers *Dpn*I and *Mbo*I before transformation of *E. coli*. *Dpn*I only cuts DNA methylated at adenine residues in GATC sequences (15), i.e., DNA synthesized in *dam*$^+$ bacteria, whereas *Mbo*I cuts nonmethylated DNA (7), in this case DNA synthesized in the animal cell. Simian cells were transfected under conditions of optimal specific infectivity for each of the three recombinant plasmids. The results (Table 2) clearly show that plasmid replication, i.e., the appearance of *Dpn*I-resistant plasmid molecules, occurred in the presence of the SV40 replication origin and SV40 T antigen; pKK17 was the only plasmid which replicated in Vero cells, as expected, since only this plasmid codes for T antigen. On the other hand, all three plasmids replicated in COS-1 cells in which T antigen was supplied exogenously.

Measurement of plasmid copy number showed that high specific infectivity of poliovirus cDNA (Table 1) could be correlated with the ability of the recombinant plasmid to replicate in simian cells (Fig. 2 and Table 2). We suggest that the high specific infectivity of our constructions and of those of other workers (22; V. Racaniello, personal communication) depends on the presence of eucaryotic replication signals rather than on the presence of promoter elements.

TABLE 2. Replication of recombinant plasmid DNA in simian cells

DNA source	Cell line and time post-transfection[a]	CFU/10^6 cells after digestion with[b]:			% Replicated molecules[c]
		No enzyme	*Dpn*I	*Mbo*I	
pKK17	Vero				
	30 min	21	1	24	4.8
	47 h	194	156	26	80
	COS-1				
	30 min	22	0	21	<4.5
	47 h	253	216	28	86
pKK25	Vero				
	30 min	11,170	60	12,060	0.5
	47 h	142	8	130	5.6
	COS-1				
	30 min	21	0	18	<4.8
	47 h	284	280	2	98.6
pSV-polio	Vero				
	30 min	9,820	130	9,470	1.3
	47 h	130	10	146	7.7
	COS-1				
	30 min	34	0	26	<2.9
	47 h	254	260	70	102
pBR322[d]	Vero	860	40	ND[e]	4.7
	COS-1	470	30	ND	4

[a] Zero time was the time of glycerol shock. DNA was extracted by the Hirt method (13) at the times indicated (see the legend to Fig. 2).

[b] Each Hirt extract was digested by the enzyme indicated, reextracted with phenol-chloroform, and then ethanol precipitated before transformation of *E. coli* 1106. Ampicillin-resistant colonies were scored, and their numbers were corrected to CFU per 10^6 simian cells and corrected for transformation efficiency with purified pBR322 DNA.

[c] *Dpn*I-resistant plasmid DNA was considered to be replicated in simian cells. The percentage of replicated molecules was calculated by comparison with undigested plasmid. By comparison with pBR322 controls, values under 5% cannot be considered to be significant.

[d] pBR322 DNA (1 ng) was added to a Hirt extract from 10^6 mock-transfected cells and treated in parallel to experimental samples.

[e] ND, Not determined.

We thank Vince Racaniello and David Baltimore for the gift of pSV-polio and Moshe Yaniv for COS-1 cells. We are grateful to Henri Agut and Sylvie van der Werf for critical reading of the manuscript.

This work was supported by Centre National de la Recherche Scientifique (CNRS, UA 545) and Ministère de la Recherche et de l'Industrie (MRI, Programme Biotechnologies).

LITERATURE CITED

1. **Benoist, C., and P. Chambon.** 1981. *In vivo* sequence requirements of the SV40 early promoter region. Nature (London) **290**:304–310.
2. **Bergsma, D. J., D. M. Olive, S. W. Hartzell, and K. N. Subramanian.** 1982. Territorial limits and functional anatomy of the simian virus 40 replication origin. Proc. Natl. Acad. Sci. USA **79**:381–385.
3. **Chou, J. Y., J. Avila, and R. G. Martin.** 1974. Viral DNA synthesis in cells infected by temperature-sensitive mutants of simian virus 40. J. Virol. **14**:116–124.
4. **Contreras, R., D. Gheysen, J. Knowland, A. van de Voorde, and W. Fiers.** 1982. Evidence for the direct involvement of DNA

replication origin in synthesis of late SV40 RNA. Nature (London) **300**:500–505.
5. **Fiers, W., R. Contreras, G. Haegeman, R. Rogiers, A. van de Voorde, H. van Heuversywn, J. van Herreweghe, G. Volkaert, and M. Ysebaert.** 1978. Complete nucleotide sequence of SV40 DNA. Nature (London) **273**:113–120.
6. **Frost, E., and J. Williams.** 1978. Mapping temperature-sensitive and host-range mutations of adenovirus type 5 by marker rescue. Virology **91**:39–50.
7. **Gelinas, R. E., P. A. Myers, and R. J. Roberts.** 1977. Two sequence-specific endonucleases from *Moraxella bovis*. J. Mol. Biol. **144**:169–179.
8. **Gluzman, Y.** 1981. SV40-transformed simian cells support the replication of early SV40 mutants. Cell **23**:175–182.
9. **Gosh, P. K., V. B. Reddy, J. Swinscoe, P. Lebowitz, and S. M. Weissman.** 1978. Heterogeneity and 5′-terminal structures of the late RNAs of simian virus 40. J. Mol. Biol. **126**:813–846.
10. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
11. **Hartzell, S. W., B. J. Byrne, and K. N. Subramanian.** 1984. Mapping of the late promoter of simian virus 40. Proc. Natl. Acad. Sci. USA **81**:23–27.
12. **Hartzell, S. W., B. J. Byrne, and K. N. Subramanian.** 1984. The simian virus 40 minimal origin and the 72-base-pair repeat are required simultaneously for efficient induction of late gene expression with large tumor antigen. Proc. Natl. Acad. Sci. USA **81**:6335–6339.
13. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
14. **Koch, G.** 1973. Interaction of poliovirus-specific RNAs with HeLa cells and *E. coli*. Curr. Top. Microbiol. Immunol. **62**:89–138.
15. **Lacks, S., and B. Greenberg.** 1975. A deoxyribonuclease of *Diplococcus pneumoniae* specific for methylated DNA. J. Biol. Chem. **250**:4060–4066.
16. **Lebkowski, J. S., R. B. Dubridge, E. A. Antell, K. S. Greisen, and M. P. Calos.** 1984. Transfected DNA is mutated in monkey, mouse, and human cells. Mol. Cell. Biol. **4**:1951–1960.
17. **Lee, Y. F., A. Nomoto, B. M. Detjen, and E. Wimmer.** 1977. The genome-linked protein of picornaviruses. I. A protein covalently linked to poliovirus genome RNA. Proc. Natl. Acad. Sci. USA **74**:59–63.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Murray, N. E., W. J. Brammer, and K. Murray.** 1976. Lamboid phages that simplify the recovery of *in vitro* recombinants. Mol. Gen. Genet. **150**:53–101.
20. **Racaniello, V. R., and D. Baltimore.** 1981. Cloned poliovirus cDNA is infectious in mammalian cells. Science **214**:916–919.
21. **Razzaque, A., H. Mizusawa, and M. M. Seidman.** 1983. Rearrangement and mutagenesis of a shuttle vector plasmid after passage in mammalian cells. Proc. Natl. Acad. Sci. USA **80**: 3010–3014.
22. **Semler, B. L., A. J. Dorner, and E. Wimmer.** 1984. Production of infectious poliovirus from cloned cDNA is dramatically increased by SV40 transcription and replication signals. Nucleic Acids Res. **12**:5123–5141.
23. **Tegtmeyer, P.** 1972. Simian virus 40 deoxyribonucleic acid synthesis: the viral replicon. J. Virol. **10**:591–598.
24. **Tooze, J. (ed.).** 1980. Molecular biology of tumor viruses, part 2. DNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Wychowski, C., S. van der Werf, and M. Girard.** 1985. Nuclear localization of poliovirus capsid polypeptide VP1 expressed as a fusion protein with SV40-VP1. Gene **37**:63–71.
26. **Yogo, Y., and E. Wimmer.** 1972. Polyadenylic acid at the 3′ terminus of poliovirus RNA. Proc. Natl. Acad. Sci. USA **69**:1877–1882.