Fragment Spanning the SV40 Replication Origin Is the Only DNA Sequence Required in cis for Viral Excision

Abstract. *A 311–base pair fragment containing the SV40 origin of replication was linked to the chicken thymidine kinase gene on a recombinant plasmid. This molecule was transfected into human 143 thymidine kinase-deficient (TK^-) cells, and colonies positive for thymidine kinase were selected. When cell lines derived from these colonies were fused to permissive simian cells that produce SV40 T antigen, the recombinant plasmid excised itself from the human cellular genome and replicated with a high copy number per cell. These results show that this segment of the viral genome is the only sequence required in cis to mediate SV40 excision and replication upon fusion to permissive cells. In addition, we have shown that excised plasmids apparently identical to the input DNA can be efficiently rescued in* Escherichia coli. *SV40 excision and replication may therefore be useful for the recovery of cloned genes from eukaryotic cells.*

Cells that are nonpermissive or semipermissive for lytic growth of SV40 can be stably transformed by the virus in culture. The resulting transformed cell lines invariably contain viral DNA sequences integrated into the cellular DNA, apparently at random sites on both the viral and cellular genomes (*1*). In nonpermissive rodent cells, this integrated DNA is the only viral DNA present in the cell, whereas in newly transformed semipermissive human cells, both integrated DNA and low levels of freely replicating viral DNA can be detected. Upon extensive passaging in culture, however, these transformed human cell lines tend to stabilize and cease to produce detectable extrachromosomal viral DNA. The integrated viral DNA in both transformed human and rodent cells can be induced to excise from the chromosome and replicate freely by fusion with permissive simian cells (*2–4*). Botchan, Topp, and Sambrook (*2*) proposed that this excision was a direct consequence of the in situ replication of viral DNA sequences and that all factors required for viral DNA replication would therefore be required for a high level of excision. They suggested that multiple rounds of DNA synthesis were initiated from a single viral origin upon fusion with permissive cells, leading to an "onion skin" of amplified viral DNA sequences (*2*). This structure would be a preferred substrate for viral excision by an unknown recombinational mechanism.

A direct prediction of the replication model of viral DNA excision is that the only DNA sequence required in cis for efficient excision-replication should be the viral origin of replication. To test this prediction, we constructed a pBR322-derived recombinant plasmid containing both the chicken thymidine kinase (ChTK) gene and the 311–base pair (bp) SV40 Eco RIIG fragment, which spans the viral origin of replication. The structure of this recombinant molecule, pBRChTKRIIG, is shown in Fig. 1A. This construct was transfected into human 143 thymidine kinase–deficient (TK^-) cells, and TK^+ colonies were selected by growth in hypoxanthine-aminopterin-thymidine (HAT) medium. Several independent colonies were picked and expanded into cell lines. To determine the physical state of the plasmid DNA in the TK^+ transfectants, we prepared total cellular DNA from several cell lines and subjected them to blot-hybridization analysis (*5*). Undigested total cell DNA was fractionated by electrophoresis in an agarose gel, blotted, and hybridized with the pBRChTK probe. Lanes a, b, and c of Fig. 1B contained plasmid reconstructions simulating 5, 0.5, and 0.05 copies of plasmid DNA per cell; lanes d to g contained 10 μg of chromosomal DNA from four independently isolated cell lines designated 143TKRIIG-2, -4, -5, and -7. No extrachromosomal supercoiled plasmid DNA was detected within a limit of up to 0.05 copy per cell. The structure of the integrated plasmid DNA was also examined in three of these cell lines (Fig. 1C). Cell DNA's from lines 143TKRIIG-2, -5, and -7 were digested with Bam HI, which cleaves the parental plasmid DNA once. Digestion of 143TKRIIG-2 DNA with this enzyme produced five prominent bands, including one doublet (lane a), none of which migrated with the linearized plasmid DNA. Digestion of this DNA with a combination of Eco RI plus Hind III gave rise to a complex array of bands, including the 4.2- and 2.2-kb bands obtained when the parental plasmid is digested with these enzymes (Fig. 2B). Although we have not shown unambiguously that these two fragments are contiguous in the integrated DNA, our ability to rescue intact plasmid DNA from this cell line indicates that this is the case (see below). We therefore believe that this cell line contains at least

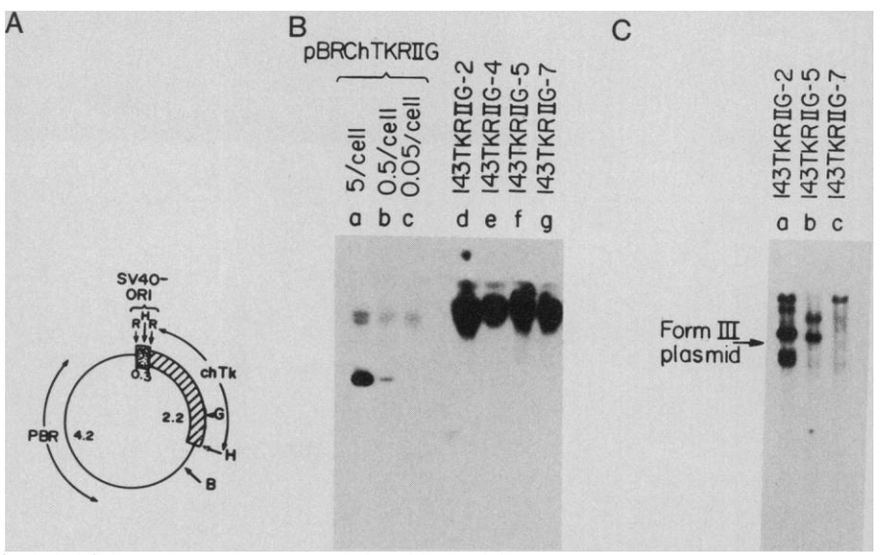

Fig. 1. (A) Schematic representation of plasmid containing 4.2 kb of pBR322, a 2.2-kb fragment containing the chicken thymidine kinase gene (*12*) and a 0.3-kb Eco RII fragment containing the SV40 origin of replication: *R*, Eco RI; *H*, Hind III; *B*, Bam HI; and *G*, Bgl II. (B) Genomic blot of undigested cell DNA's from cell lines transformed by pBRChTKRIIG. A DNA–calcium phosphate precipitate was formed with mouse TK^- DNA as carrier (*16*). This precipitate was added to human 143 TK^- cells, and TK^+ transfectants were selected by growth in HAT medium. Total cellular DNA was prepared from four independent TK^+ cell lines and fractionated on an agarose gel; the products were hybridized with pBRChTK probe. Lanes a to c are plasmid reconstructions simulating 5.0, 0.5, and 0.05 copies of plasmid per cell. The three bands seen represent forms I, II, and III of the plasmid. Lanes d to g contain 10 μg of undigested DNA from the four independent cell lines. (C) Genomic blots of digested cellular DNA's. Cellular DNA (10 μg) was digested with Bam HI, fractionated by electrophoresis in an agarose gel, transferred to nitrocellulose by the method of Southern (*5*), and hybridized with ^{32}P-labeled pBRChTK.

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one partial tandem duplication of plasmid sequences. DNA from 143TKRIIG-5 gave two bands upon Bam HI digestion (lane b), one of which migrated with the linear form of the plasmid. Digestion of 143TKRIIG-5 DNA with two additional enzymes (Pst I and Bgl II) that cleave the plasmid once also produced a unit length fragment (data not shown). Since no extrachromosomal plasmid DNA was detected in this cell line (Fig. 1B), we conclude that the integrated plasmid DNA in this cell contains a tandem duplication of a large portion of the plasmid molecule.

Digestion of 143TKRIIG-7 DNA with Bam HI gave rise to a single high molecular weight fragment (lane c), indicating that a single insert of plasmid DNA lacking the Bam HI site is probably integrated into this cell line. Digestion of this cellular DNA with either Bgl II or Hind III, each of which cuts the plasmid once, confirms that there is a single insert of plasmid DNA in this cell line. When DNA from the parental cell line 143 TK^- is digested with these restriction enzymes and hybridized with pBRChTK probe, no specific hybridizing bands are seen (data not shown). In summary, the genomic blot analyses of these representative cell lines show that the plasmid DNA does not persist extrachromosomally and that the structures of the integrated plasmid DNA's are characteristic of those seen in SV40-transformed cells.

To determine whether the integrated plasmid DNA's in these cell lines could excise and replicate when both T antigen and permissive factors were provided in trans, we utilized the cell line COS7. This simian cell line has been transformed by an origin-defective mutant of SV40 (*6*) and constitutively produces a level of T antigen sufficient to support replication from an SV40 origin. Cells from lines 143TKRIIG-2, -5, and -7 were fused with COS7 cells by use of polyethylene glycol (*7*). Forty-eight hours after fusion, the cells were lysed, and low molecular weight DNA was prepared from Hirt supernatants (*8*). When this DNA was subjected to electrophoresis on an agarose gel and hybridized with a plasmid probe, each of the three cell lines showed a distinctive pattern of excision products (Fig. 2A). Cell line 143TKRIIG-5 contained extrachromosomal DNA exclusively at the positions of forms I and II plasmid markers (lane b in Fig. 2A), indicating that a precise excision of unit length plasmid molecules had occurred. This is not surprising, since the genomic blots had suggested that 143TKRIIG-5 contained a partial tandem duplication of plasmid sequences, and homologous recombination within such a tandem repeat would give rise to unit length excision products. Cell line 143TKRIIG-2 gave rise to both precise excision products and a heterogeneous smear of hybridizing material (lane a), whereas 143TKRIIG-7 gave only a heterogeneous smear (lane c). We generalized these results by fusing more than 20 human TK^+ cell lines containing integrated copies of the plasmid pBRChTKRIIG to COS7 cells and examining the excision products by hybridization. In approximately 50 percent of these fusions, we detected excised DNA that was indistinguishable from the input plasmid. We propose that the high frequency of precise excision products is due to the frequent occurrence of tandem duplications after transfection with supercoiled plasmid DNA's, an effect that was first noted by Hanahan *et al.* (*9*).

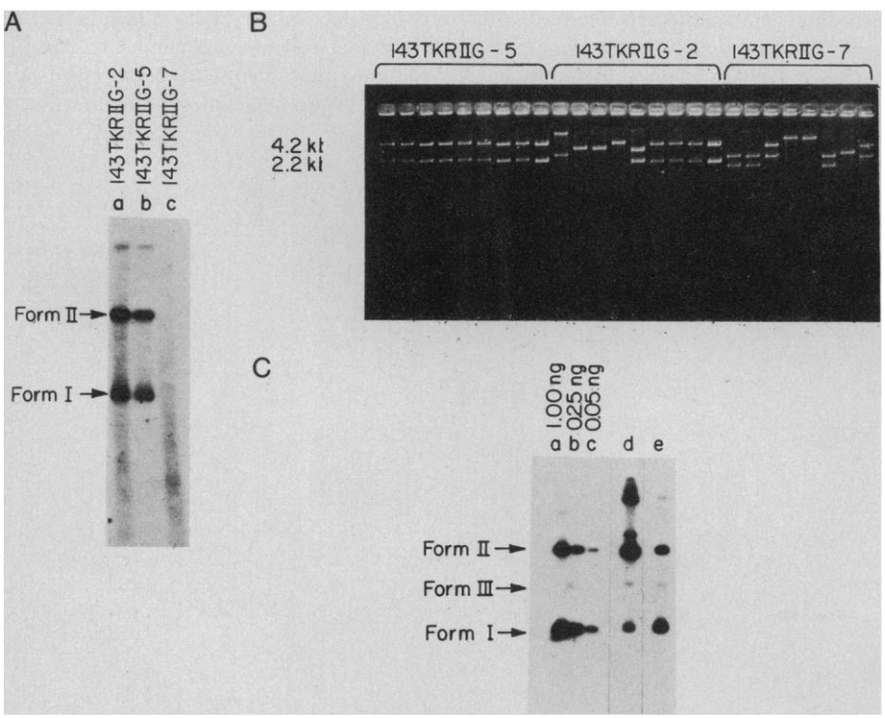

Fig. 2. (A) Blot-hybridization of excised DNA's. Cell lines 143TKRIIG-2, -5, and -7, which contain integrated pBRChTKRIIG DNA, were fused with COS7 cells by the use of polyethylene glycol (*2*, *7*); Hirt supernatants were collected after 48 hours. DNA was run on an agarose gel, blotted, and hybridized to a pBRChTK probe. The positions of forms I and II of the parental plasmid are indicated. (B) Restriction digests of excised plasmids isolated in *E. coli* HB101. The Hirt supernatant DNA's shown in (A) were transformed into *E. coli* HB101, and amp^r colonies were selected. Plasmid DNA was prepared from each colony and digested with a mixture of Eco RI and Hind III. This treatment digests the parental plasmid pBRChTKRIIG into three fragments of 4.2, 2.2, and 0.3 kb (Fig. 1A). The 0.3-kb fragment is not visible on this gel. Representative excision products from the three cell lines are shown. (C) Quantitation of replicating plasmid DNA in Hirt supernatants of fused cells. The amount of plasmid DNA in Hirt supernatants after fusion to COS7 cells was estimated by comparison to plasmid reconstructions (lanes a to c). The Hirt supernatant DNA from approximately 4×10^5 fused cells is shown in lanes d and e. The positions of forms I, II, and III plasmid DNA are indicated.

We further analyzed the excision products from each fusion by recloning them in *Escherichia coli*. DNA from Hirt supernatants of fused cells was transformed into *E. coli* HB101, and 64 amp^r (ampicillin-resistant) colonies from each transformation were selected. Plasmid DNA was prepared from each of the selected colonies, and the restriction pattern was compared to that of the parental plasmid pBRChTKRIIG after digestion with Eco RI plus Hind III. As expected, cell line 143TKRIIG-5 gave rise exclusively (64/64) to plasmids that were identical to the input pBRChTKRIIG DNA (Fig. 2B). Transformants from line 143TKRIIG-2 contained plasmids identical to (32/64) as well as plasmids different from (32/64) the parental plasmid, whereas those from 143TKRIIG-7 contained only altered plasmids. The patterns of plasmids rescued in *E. coli* therefore paralleled the patterns seen on agarose gels of Hirt supernatant DNA's. Hirt extracts from unfused cells did not give rise to any colonies in *E. coli*, again suggesting that there was no preexisting "free" DNA in these cells.

Cell lines that gave rise to precise excision products contained the greatest amount of plasmid DNA in Hirt superna-

tants (Fig. 2A). We propose that this occurs because these cell lines contain tandem duplications of plasmid sequences and that such duplications are preferred substrates for recombinational excision. To determine the optimal efficiency of the excision-rescue process, two such cell lines were fused to COS7 cells, and the amount of free plasmid in Hirt supernatants was estimated by plasmid reconstruction experiments (Fig. 2C). Lanes a, b, and c in Fig. 2C contained 1.0, 0.25, and 0.05 ng of plasmid DNA marker; lanes d and e contained 20 percent of the Hirt supernatant DNA from approximately 2×10^6 cells of two independent cell lines fused to COS7 cells. These experiments show that in approximately 50 percent of the cell lines examined, one to several nanograms of the input plasmid DNA can be isolated from approximately 10^6 fused cells. This is equivalent to the levels of excised DNA seen with bona fide SV40 transformants and is sufficient to give rise to several thousand colonies when the DNA is used to transform *E. coli* HB101 to amp^r.

Our results demonstrate that sequences directly surrounding the SV40 origin of replication are the only viral DNA sequences required in cis to mediate SV40 excision-replication after fusion to permissive simian cells. The following control experiments support this contention. No extrachromosomal plasmid DNA is detected when human cell lines transformed by pBRChTK are fused with COS7 cells or when any of the human TK$^+$ transformants are fused with permissive simian cells that do not produce SV40 T antigen. Plasmid sequences are not supplying functions important for excision and replication, since cell lines transformed by fragments of SV40 removed from the plasmid vector yield detectable replicating forms after fusion with COS7 cells (*10*). Finally, small deletions at the Bgl I site defining the origin of replication, which inactivate neither the early promoter nor T antigen binding, yield SV40 molecules capable of integration and stable transformation, but inactive for excision (*11*). This implies that it is the replication origin itself within the Eco RIIG fragment that is important for excision.

The patterns of excision products seen here (that is precise, random, or mixtures) parallel that seen with virally transformed cell lines, where the presence of tandem duplications was thought to be due to low levels of DNA replication during viral infection. It is unlikely that replication is occurring in our experiments, since the recombinant plasmid used contains no A gene. We conclude that the tandem duplications observed here, and perhaps those seen during viral transformation, occur by a recombinational mechanism. Recombination and "pekelasome" formation during DNA transfection have been reported (*12*).

The small size (0.3 kb) of the sequence required, and the ease and efficiency with which the input DNA can be recovered in *E. coli*, will make this a useful "shuttle" system for the recovery of cloned DNA sequences from mammalian cells. In addition, the simplicity of the viral sequences required may be important in minimizing the effects of the vector on the expression of a cloned gene. Since the origin is inactive in the absence of T antigen, and since the promoter shows only low levels of activity in the absence of an activator element, it is unlikely that the viral sequences will affect the expression of a linked gene. In some situations this may be preferable to other "shuttle" systems such as that provided by bovine papillomavirus (BPV) (*13*) where many of the viral coding and regulatory sequences are present.

We envision several possible applications of this "shuttle" system. A cloned gene linked to an SV40 origin could be introduced into mammalian cells, mutants selected in the phenotype conferred by that gene, and the mutated genes easily recovered by fusion with COS7 cells followed by transformation of *E. coli*. Alternatively, an SV40 origin could be introduced into a eukaryotic cloning vector so that once a gene had been identified it could be easily isolated in *E. coli*. Finally, if a gene could be introduced into the germ line of an animal (*14, 15*) its structure could be examined in many differentiated tissues by SV40-mediated excision and rescue.

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Simultaneous Expression of Globin Genes for Embryonic and Adult Hemoglobins During Mammalian Ontogeny

Abstract. *The dominant hemoglobin of the adult hamster was detected in yolk-sac erythroid cells, and its identity was confirmed by peptide mapping and by analysis of relevant peptides. Both the presence and active synthesis of two embryonic hemoglobins presumed to exist only in yolk-sac erythroid cells were detected in neonatal liver and spleen. Thus the time span of expression of both embryonic and adult globin genes during mammalian ontogeny may be considerably broader than presently believed.*

Early in normal mammalian ontogeny, the most primitive erythroid cells (yolk-sac cells) of the embryo synthesize an "embryonic" set of hemoglobins, whereas in ontogenically later erythroid organs (liver, spleen, and finally bone marrow) an "adult" set of hemoglobins is made. This temporal progression in globin gene expression is a well-studied phenomenon of developmental biology. However, reports conflict on whether embryonic and adult hemoglobins can coexist in cells from the same erythropoietic organ. For example, Fantoni *et al.* (*1*) and Barker (*2*) detected only embryonic hemoglobins in yolk-sac cells and only adult hemoglobins in fetal liver cells of mice. Similar results were obtained by Bruns and Ingram (*3*) and by Beaupain *et al.* (*4*) for avian species. On the other hand, there is evidence for the coexistence of embryonic and adult he-

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