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Analysis of Nonpermissivity in Mouse Cells Overexpressing Simian Virus 40 T Antigen

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To analyze the nature of the nonpermissivity of mouse cells for simian virus 40 (SV40) DNA replication, we isolated mouse cells producing SV40 T antigen (Tag) at levels equal to or greater than that found in COS1 cells. These mouse cells were nonpermissive for the replication of exogenously added SV40 DNA, although purified Tag isolated from these cells was able to support SV40 DNA replication in vitro. Furthermore, when mouse cells expressing Tag were fused to monkey cells, SV40 DNA replication was observed. These results indicate that the mere production of large quantities of wild-type SV40 Tag does not overcome the block of nonpermissivity in mouse cells and that cellular factors must play a critical role.

The replication of simian virus 40 (SV40) DNA in permissive monkey kidney cells requires both *cis*-acting DNA sequences and *trans*-acting factors. The DNA sequences containing the origin of replication (*ori*) have been localized to a single region of the viral genome near the *Bgl*I site (4). While numerous *trans*-acting protein factors, including histones and enzymes, that are required for the initiation, elongation, and termination of DNA synthesis are provided by the host cell, a product of the virus early gene, large T antigen (Tag), is the only virus-encoded *trans*-acting factor necessary for SV40 replication. Tag acts on two levels to affect the SV40 DNA replication process: (i) it is required for the initiation of each round of replication (31), and (ii) it stimulates the synthesis of host cell factors involved in replication, such as deoxypyrimidine kinases, DNA ligase, DNA polymerase, and nonhistone chromosomal proteins (33). Graessman et al. (11) demonstrated that productive infection only occurs in cells that contain more than the "threshold" level of Tag necessary to induce cellular functions and to interact with the SV40 *ori*. Furthermore, we have previously demonstrated that the amount of Tag in permissive monkey cells is the limiting factor governing the extent of SV40 DNA replication (6).

Infection of mouse cells with SV40 results in the expression of Tag and the induction of cellular DNA synthesis, but the cells do not replicate the viral DNA or produce late capsid proteins; that is, they are nonpermissive. A small proportion of the infected cells will be stably transformed and express both large (T) and small (t) tumor antigens from an integrated copy(ies) of the viral genome (33). The integrated viral genome can be rescued by fusion of such SV40-transformed murine cells with permissive monkey cells (7, 17) or enucleated, permissive cytoplasts (3, 24). It is also possible to rescue viral genomes by injecting permissive-cell cytoplasm into such murine cells (A. Graessman, personal communication). From these and other results, it would seem that excision and extrachromosomal replication of SV40 DNA depend on a diffusible factor supplied by permissive cells. The recently published results that primate but not mouse DNA polymerase α-primase complex was able to support SV40 DNA replication in vitro (23) would suggest that polymerase α-primase complex is either the only or one of the missing permissivity factors.

There are three possible explanations for the inability of SV40 DNA to replicate in murine cells: (i) they lack a permissivity factor which is present in permissive monkey cells and participates directly in SV40 DNA replication or is responsible for proper modification of Tag; (ii) they contain an inhibitor of SV40 replication which is inactivated after fusion with monkey cells; or (iii) the quantity of SV40 Tag produced in murine cells is insufficient to allow SV40 DNA replication.

Data supporting the third possibility have been published by Graessmann et al. (11, 12). These authors reported that after microinjection of large quantities of SV40 DNA, mouse 3T3 cells produced large quantities of Tag as well as late viral proteins and virus progeny. To test this hypothesis further, as well as to analyze whether Tag produced in mouse cells is active in an in vitro DNA replication assay, a series of mouse cell lines transformed with bovine papillomavirus (BPV) Tag expression vectors were isolated, and the properties of these cells are described in this paper.

MATERIALS AND METHODS

General methods. Preparation of plasmid DNA, restriction enzyme digestion, ligation, and bacterial transformation were performed by standard procedures (22).

BPV vector constructions. Plasmid pPX (obtained from J. Sambrook) was used as an intermediate to cassette *Xho*I-*Bam*HI fragments containing the different transcription units for the expression of Tag into the BPV vector. It contains the 2.8-kilobase (kb) *Hpa*II-*Bam*HI fragment of the SV40 early region cloned into the *Cla*I-*Bam*HI vector in pXf3, with the *Hpa*II-*Cla*I junction converted to an *Xho*I site by ligation of synthetic linkers (25). The large *Kpn*I-*Bam*HI vector fragment of pPX was ligated to *Kpn*I-*Bam*HI fragments containing the SV40 early region from either pK1*dl*26 or pKMT9 (6) to generate pPX*dl*26 and pPXMT, respectively. pPX*dl*26 contains a deletion of SV40 nucleotide pair (np) 5212-9 and an insertion of three *Bgl*II linkers that disrupt the SV40 origin of replication. pPXMT contains the mouse metallothionein I *Kpn*I-*Bgl*II promoter fragment fused to SV40 np 5195 in lieu of the SV40 promoter.

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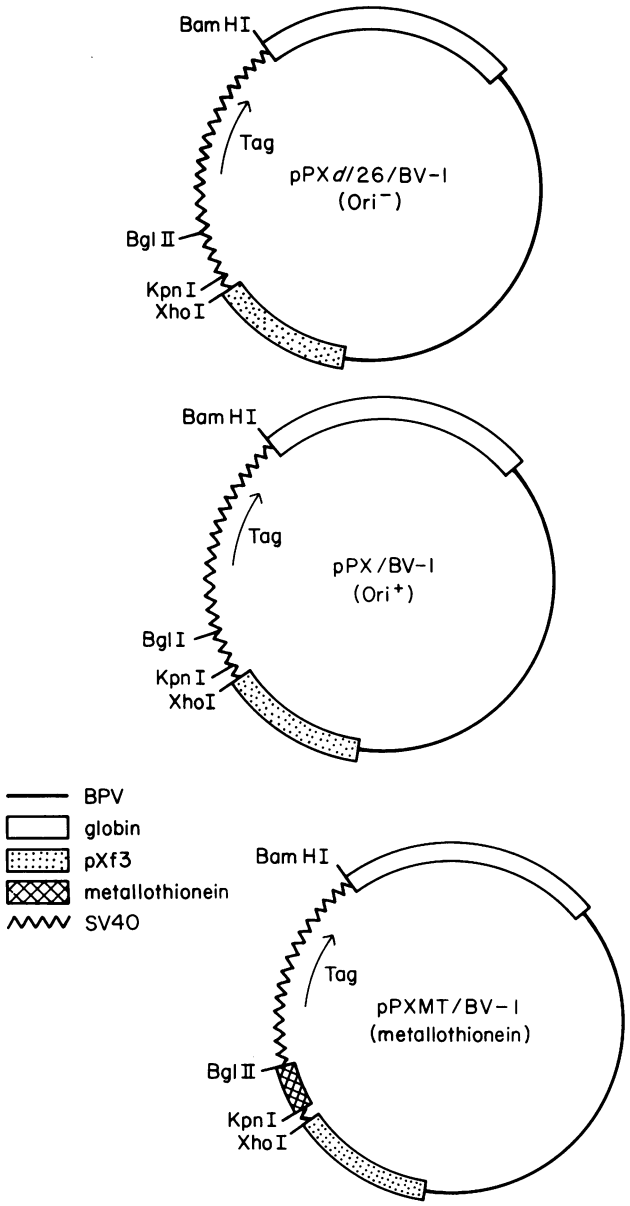

FIG. 1. Structures of the BPV-Tag expression vectors. Details of the construction are given in Materials and Methods. The only difference among the three constructs is the structure of promoters driving the expression of SV40 Tag: pPX/BV-1 (*ori*$^+$) has the wild-type SV40 early region; pPX*dl*26/BV-1 (*ori*$^-$) has the SV40 early promoter but without the SV40 origin sequences; and pPXMT/BV-1 has a metallothionein promoter replacing the SV40 early promoter.

The structures of the BPV vectors expressing SV40 Tag are shown in Fig. 1. The plasmid pPX/BV1 (obtained from J. Sambrook) is the BPV vector containing the wild-type SV40 early region used in these studies (26). It was derived by ligating the *Sal*I-*Bam*HI fragment of pBPV-BV1 (34) that contains both the 5.5-kb subgenomic transforming fragment of BPV DNA and a segment of human β-globin DNA to the large *Sal*I-*Bam*HI fragment of pPX containing the bacterial vector sequences and the SV40 *ori*$^+$ transcription unit for Tag. BPV vectors pPX*dl*26/BV1 and pPXMT/BV1 were derived from pPX/BV1 via substitution of the *Xho*I-*Bam*HI cassettes containing the Tag genes from pPX*dl*26 and pPXMT, respectively.

Construction of the replication test plasmid pK1K1. Plasmid pK1K1, containing a 1.7-kb internal duplication of the SV40 late region sequences, was constructed to test for the replication in vivo of SV40 DNA. The parental plasmid pK1 contains the complete SV40 genome cloned via the *Eco*RI site into the vector pMK16-6 (9). pK1 was linearized by partial digestion with *Cla*I and completely digested with *Bam*HI. The *Hpa*II-*Bam*HI fragment of SV40 spanning the late coding sequences (np 346 to 2533) was ligated to the *Cla*I-*Bam*HI-digested pK1 DNA, thus duplicating the region in the resulting plasmid, pK1K1. Other studies (14) have demonstrated the ability of this molecule to undergo homologous recombination in vivo to generate functional viral genomes.

Growth and transfection of cells. Cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2 at 37°C. Transformation of C127 cells was accomplished by using 1 μg of BPV vector in a $CaPO_4$ coprecipitation with 20 μg of HeLa cell carrier DNA (5). Following a 7-h transfection, cells were boosted with 20% dimethyl sulfoxide (DMSO) in DMEM for 3 min at room temperature, washed thoroughly with DMEM to remove DMSO, and cultured in DMEM containing 5% FBS. Transformants were selected on the basis of morphological phenotype 2 weeks after transfection.

A line of C127 mouse cells transformed with pPXMT/BV1 was subjected to cloning in soft agar to select for anchorage-independent cell growth. Colonies obtained by this method were transferred back to monolayers in DMEM containing 10% FBS to expand the cell culture. Cells were subsequently grown for 1 to 2 passages in F13 medium (Gibco) containing 10% FBS on petri dishes, detached from the plastic surface with a stream of medium, and grown in spinner culture in the same medium. These cells were used as a source of preparative quantities of purified mouse Tag.

Transfection of pK1K1 DNA for replication experiments was accomplished by DEAE-dextran as described previously (6) with the following modifications. Cells grown in 60-mm culture dishes were transfected with 100 ng of pK1K1 DNA for 1 h with 250 μg of DEAE-dextran per ml. The cells were subsequently incubated for 3 h in DMEM containing 10% FBS and 80 μM chloroquine hydrochloride (21). Cell cultures were maintained in DMEM containing 10% FBS either with or without added heavy metals (100 μM $ZnCl_2$ and 1 μM $CdSO_4$) to induce the metallothionein promoter.

Analysis of Tag. The methods for pulse-labeling cells with [^{35}S]Methionine, extracting Tag, immunoprecipitating labeled Tag with monoclonal antibody, analyzing immunoprecipitates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and radioimmunoassay of Tag have been described (6). Radioimmunoassay was made quantitative by comparison with a standard curve of purified mouse Tag of known concentration.

Cell fusion. BPV Tag-transformed mouse cell lines were split 1:4 and coplated with an approximately equal number of C6 monkey cells (10) in DMEM containing 10% FBS in 100-mm dishes. On the following day, cells were fused by treatment with 50% (wt/vol) polyethylene glycol 1000 in DMEM at room temperature for 1.5 min. Monolayers were washed twice with 15% DMSO in DMEM, once with DMEM, and once with DMEM containing 2% FBS before culturing in DMEM containing 5% FBS. After 2 days, low-molecular-weight DNA was prepared (15) and analyzed

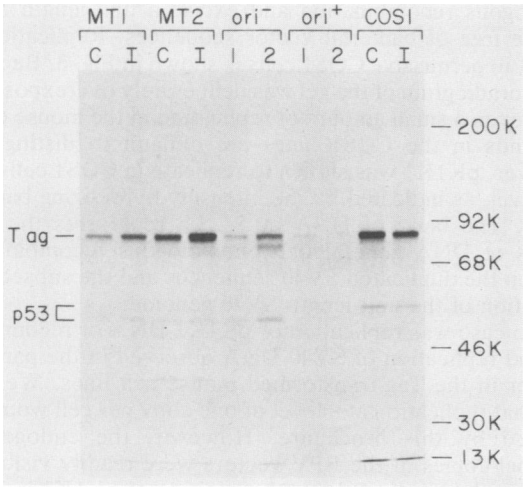

FIG. 2. Synthesis of Tag by transformed mouse cell lines. [^{35}S]methionine-labeled cell extracts from the indicated cell lines were immunoprecipitated with PAb416 monoclonal antibody to Tag and protein A-Sepharose. Both control (C) and induced (I) cultures of COS1, MT1, and MT2 cells were analyzed. Only control cultures of ori$^-$1, ori$^-$2, ori$^+$1, and ori$^+$2 cells were analyzed. An amount of immunoprecipitated protein equivalent to that from 2×10^5 cells was applied to a 10% sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, the gel was dried and exposed directly to X-ray film. Molecular weights (in thousands) are indicated to the right.

for the presence of SV40 sequences by Southern blotting (28). Parental C127 mouse cells and COS1 monkey cells (8) served as negative and positive controls, respectively, in the fusion experiment.

In vitro replication of SV40 DNA. A recombinant adenovirus vector (AdSVR115) that overproduces SV40 Tag in infected human 293 cells has been described (30). Tag was also prepared from a line of transformed mouse cells adapted to spinner culture as described above. Both Tags were purified by the immunoaffinity procedure (27) with PAb419 (13) coupled to protein A-Sepharose. DNA synthesis experiments were performed with cytosol extracts from human 293 cells (30) adjusted to 0.1 M NaCl and centrifuged at 100,000 $\times$ *g* for 1 h. Equivalent amounts of human and mouse Tag, as judged by polyacrylamide gel electrophoresis, were included in the in vitro replication experiments.

RESULTS

Transformation of C127 mouse cells with BPV-Tag expression vectors. The BPV expression vectors used to transform C127 mouse cells are shown in Fig. 1. These vectors contain (i) the 69% *Hin*dIII-*Bam*HI fragment of BPV DNA responsible for murine cell transforming functions (19) and episomal maintenance functions (20), (ii) a fragment of human DNA derived from the β-globin gene cluster which seems to stabilize episomal BPV vectors in some systems (5, 25), (iii) bacterial sequences for the propagation of the plasmid in *Escherichia coli*, and (iv) promoter and structural gene sequences for the expression of SV40 Tag. Three different promoter configurations were used in this study. Two constructs contain the SV40 early promoter with either a wild-type origin of replication (pPX/BV1) or a defective origin obtained by deletion of sequences in Tag-binding site II (pPX*d*l26/BV1). The third (pPXMT/BV1) contains the mouse metallothionein I promoter previously demonstrated to direct the high-level synthesis of Tag in response to heavy metals (6).

C127 cells transfected with the BPV-Tag expression vectors gave rise to foci of morphologically transformed cells (at a frequency of ~300 foci per μg of plasmid DNA) which were picked after 2 weeks and expanded into cell lines. All colonies displayed a transformed phenotype characteristic of BPV-transformed cells. However, the extent of the transformed phenotype was dependent on the promoter used to express Tag. The phenotype was most pronounced in colonies transformed with pPXMT/BV1, containing the mouse metallothionein promoter.

Expression of Tag by transformants. Clones were screened by indirect immunofluorescent staining for the presence of Tag. Staining revealed that almost every cell in these lines expressed Tag. In addition, induction of the metallothionein promoter in MT cell lines for 2 days by heavy metals resulted in cells that were stained more intensely for Tag than cells maintained in normal medium. Qualitatively, the intensity of fluorescence was greatest in the MT cell lines (particularly when induced by heavy metals), intermediate in the *ori*$^-$ transformants, and weakest in the *ori*$^+$ lines. Only 1 clone of 21 was defective for the expression of Tag.

Southern blotting of low-molecular-weight Hirt DNAs revealed the presence of approximately 10 to 100 copies of the vectors, which were present both as free monomers (in 18 of 21 clones) and as concatenated oligomers (data not shown). Clonal variation in the copy number was observed among the different cell lines (see Fig. 3), but this variation was not related to the particular BPV vector used to transform the cells. Restriction enzyme digestion revealed a pattern of DNA fragments identical to that of the input DNA for all clones except the one that did not express Tag. This clone contained episomal DNA which had deleted a segment of the genome containing the Tag gene. Clones transformed by pPXMT/BV1 DNA were designated MT1 and MT2, by pPX*d*l26/BV1 ori$^-$1 or ori$^-$2, and by pPX/BV1 ori$^+$1 and ori$^+$2. These clones were chosen for further study.

The level of Tag expression was quantitated by pulse-labeling transformed cell cultures with [^{35}S]methionine and gel electrophoresis of cell extracts immunoprecipitated with monoclonal antibody to Tag (Fig. 2). The analysis of mouse cell extracts revealed the presence of Tag of the same molecular size as that present in COS1 cells. Quantitation of the rate of Tag synthesis demonstrated that clones MT1 and MT2 expressed Tag at a twofold-higher rate on a per cell basis when propagated in medium containing heavy-metal

TABLE 1. Tag synthesis

Cell line	Rate of Tag synthesis[a]			Tag content[b] (μg/10^6 cells)		
	Control (C)	Induced (I)	Ratio, I/C	Control (C)	Induced (I)	Ratio, I/C
MT1	0.19	0.41	2.2	0.11	0.40	3.6
MT2	0.56	1.1	2.0	0.39	1.1	2.8
ori$^-$1	0.12	ND[c]	—[d]	0.08	ND	—
ori$^-$2	0.34	ND	—	0.12	ND	—
ori$^+$1	0.05	ND	—	<0.01	ND	—
ori$^+$2	0.05	ND	—	<0.01	ND	—
COS1	1.0	0.61	0.6	0.17	0.13	0.8

[a] Quantitative estimates of the rates of synthesis in the different cell lines were obtained by scanning densitometry of the autoradiogram shown in Fig. 2. The relatives rates of Tag synthesis were compared with that in COS1 cells, which was arbitrarily set at 1.0.

[b] Micrograms of Tag per 10^6 cells, determined by radioimmunoassay and comparison with a standard curve of purified Tag. The same cell extracts were used to determine both the rate of synthesis and the total amount of Tag.

[c] ND, Not determined.

[d] —, Not applicable.

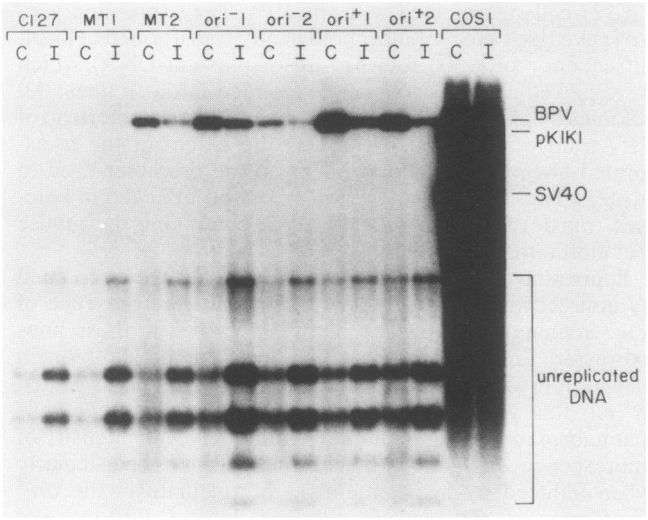

FIG. 3. Replication of SV40 in vivo. The indicated cell lines were transfected with pK1K1 DNA to test for the replication in vivo of SV40 *ori* sequences. Following transfection, cultures were incubated in either the presence (I) or absence (C) of heavy metals to induce synthesis of Tag from the metallothionein promoter. Hirt supernatant DNAs prepared 48 h later were digested with a mixture of *Bam*HI and *Dpn*I to convert all replicated plasmid to either a 9.5-kb from (pK1K1) or a 5.4-kb form (SV40) and to degrade all unreplicated plasmid to small fragments. Digests were run on a 1.1% agarose gel, blotted to nitrocellulose, and probed with a nick-translated plasmid DNA containing the early region of SV40. The migration position of the endogenous episomal copies of the BPV-Tag expression vectors is indicated (BPV).

inducers of the metallothionein promoter (Table 1). The induced rates of synthesis were comparable to those in permissive COS1 cells. By comparison, the *ori*$^-$ clones synthesized three- to eightfold less Tag than COS1 cells, and the *ori*$^+$ clones synthesized 20-fold less. None of the clones transformed with a BPV vector containing an SV40 promoter demonstrated a change in the rate of Tag synthesis after exposure to heavy metals. The gel also shows the specific immunoprecipitation of the p53 protein in both the mouse and monkey cell lines.

The same cell extracts used to quantitate the rate of Tag synthesis were assayed for total Tag protein by quantitative radioimmunoassay (Table 1). The quantity of Tag accumulating in a cell line reflected the rate of Tag synthesis by that cell line. After 2 days of induction, both MT clones accumulated threefold more Tag than uninduced controls on a per-cell basis. These levels were equal to or greater than those present in permissive COS1 cells. The ori$^-$2 clone also accumulated amounts of Tag similar to those in COS1 cells as a result of its high rate of synthesis. The accumulation of Tag by mouse cells was greater than that by COS1 cells for equivalent rates of synthesis. This may reflect differences in the stability of the protein in mouse and monkey cells. In this respect it is interesting that different clones obtained by transfection with the same BPV vector showed clonal variation in both the accumulation and rate of synthesis of Tag.

Lack of replication of SV40 DNA in transformed mouse cells. Transformed mouse cell lines were tested for their ability to support the replication of transfected plasmid DNA containing an SV40 origin. The plasmid pK1K1 was designed for this purpose. It contains a 1.7-kb duplication of the SV40 late region which has the potential to undergo homologous recombination and excise a unit-length SV40 genome free of bacterial vector sequences. Replication of pK1K1 in permissive COS1 cells is shown in Fig. 3. Because the autoradiogram of the gel was deliberately overexposed to reveal even a small amount of replication in the mouse cells, the bands in the COS1 lanes are difficult to distinguish. However, pK1K1 was shown to replicate in COS1 cells to a high level, as indicated by the intensely hybridizing band at 9.5 kb. The band of DNA at 5.2 kb represents the new species of DNA generated by homologous recombination between the duplicated SV40 sequences and the subsequent replication of the unit-length SV40 genome.

In no case was replication of pK1K1 DNA or recombination and replication of SV40 DNA observed in the parental C127 or in the Tag-transformed mouse cell lines. We estimate that replication at a level of one copy per cell would be detected by this procedure. However, the endogenous episomal copies of the BPV vectors were readily visible as intact bands migating above the position of pK1K1 in lanes containing Hirt DNA extracted from transformed mouse cells. The amount of BPV vector DNA appeared to decrease in cultures induced with heavy metals. This decrease can be attributed to cell death due to the combined effects of transfection and treatment with heavy metals. Heavy metals did not appear to have direct effects on SV40 DNA replication, at least not in monkey cells (Fig. 3) (6). We confirmed that SV40 was incable of replicating in the transformed mouse cell lines both by transfection of other SV40 origin-containing plasmids with the *Dpn*I-resistance assay for DNA replication and by infection with a recombinant adenovirus vector containing an SV40 origin (data not shown). Therefore, high-level expression of Tag was not sufficient to render mouse cells permissive for SV40 replication.

Tag synthesized by transformed mouse cells is functional in vivo and in vitro. To verify that the Tag produced in the transformed mouse cells was functional for SV40 replication, we fused the different transformed lines to C6 monkey cells. C6 cells are CV-1 monkey cells which were transformed with UV-irradiated SV40 (10). They contain a functional, integrated copy of the SV40 origin of replication. However, the *ori* does not replicate because the Tag produced by the C6 cell is defective. When fused to cells containing a replication-competent Tag, the SV40 origin will excise from the chromosome and replicate as an episomal species to high copy number (8).

Figure 4 demonstrates the excision and replication of the SV40 *ori* from the C6 cells after they were fused to the various transformed mouse cell lines. Three days after cell fusion, low-molecular-weight Hirt DNA was prepared and analyzed by Southern blotting. *Hpa*I and *Sal*I digests were performed to distinguish between replicating C6 and endogenous BPV-SV40 hybrid vector molecules. The result of fusion of C6 and COS cells is shown as a positive control. The *Hpa*I DNA fragment of 2,010 base pairs (bp) is derived from the origin of the SV40 genome (np 3733 to 5243/1 to 499) which was integrated in the C6 monkey chromosome. Its presence in Hirt supernatants is indicative of rescue and replication of the SV40 *ori* by cell fusion. The 1,067 bp fragment (SV40 np 2666 to 3733) was also generated by excision and replication (COS lane); however, the presence of this DNA in Hirt supernatants is not diagnostic of rescue of the SV40 origin from C6 cells because the endogenous BPV vector also contains this fragment. The additional bands in the lanes corresponding to transformed mouse cell lines were also derived from the endogenous coipes of the BPV vector. The Tag synthesized by the transformed mouse

cells was therefore competent to replicate SV40 *ori* sequences when supplied in *trans* by cell fusion.

Although variation in the efficiency of cell fusion from one culture to the next makes quantitation difficult, the extent of replication appeared to depend on the level of expression of Tag in the mouse cells. The MT mouse lines replicated the SV40 origin in C6 cells to a greater extent than did the ori^- lines, presumably as a result of the higher level of Tag in the MT lines. Similarly, SV40 replication was difficult to detect when C6 cells were fused to the ori^+ cells because of the low levels of synthesis of Tag by these lines. C127 cells lacking Tag showed no replication of SV40 sequences when fused to C6 monkey cells.

We also tested the ability of the mouse Tag to support the replication in vitro of SV40 DNA (30). With purified Tag from the MT2 cell line, the efficient in vitro replication of SV40 DNA could be demonstrated in a crude cell extract from permissive human 293 cells (Fig. 5). An increase in the level of incorporation of radiolabeled precursor representing SV40 replication was dependent on the addition of purified Tag prepared from either 293 or MT2 cells. The lower level of DNA replication observed for the mouse Tag may be a consequence of the lower level of purity of the protein isolated from this source.

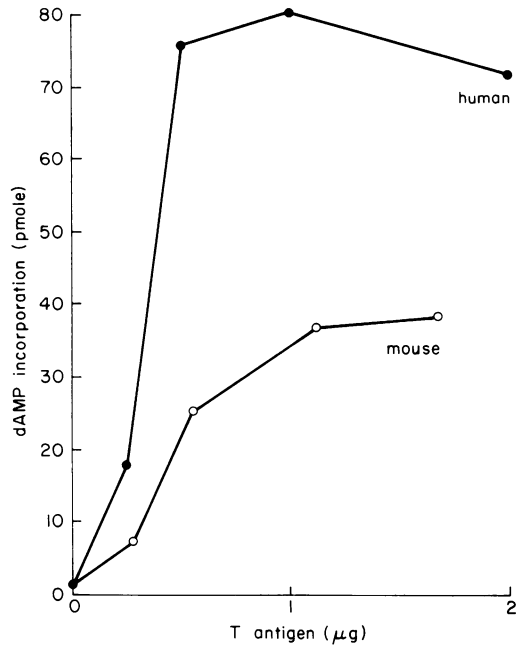

FIG. 5. Replication of SV40 in vitro by purified Tag. Tag was purified by immunoaffinity chromatography (27) from either human 293 cells infected with an adenovirus vector which overexpresses SV40 T antigen (30) or from mouse MT2 cells. Increasing amounts of purified protein were included in the in vitro SV40 DNA replication system containing 300 ng of pSV40 (30) and were incubated for 1 h.

DISCUSSION

SV40 replication is a multicomponent system requiring both *cis*-acting DNA sequences and *trans*-acting factors. The only virus-encoded *trans*-acting factor is Tag, which is necessary for the initiation of each round of viral DNA replication (32). We previously demonstrated that the amount of Tag in premissive monkey cells is the limiting factor governing the extent of SV40 DNA replication (6). Others (11, 12) have shown that SV40 late proteins are synthesized only in monkey cells which have attained a threshold concentration of intracellular Tag. In mouse cells, the level of synthesis of Tag by SV40 is normally much lower than in permissive monkey cells (11). To check whether the low level of synthesis of SV40 Tag in mouse cells is responsible for the nonpermissivity phenomenon, we overexpressed SV40 Tag in mouse cells by using an episomal vector derived from BPV. The level of expression of the protein from the different vectors was a function of the different promoters used to drive expression. The mouse metallothionein I promoter was more efficient than the ori^- SV40 early promoter, which was in turn more efficient than the ori^+ SV40 early promoter. The increased efficiency of the ori^- promoter can be attributed to the lack of autoregulation of Tag expression (32) caused by the deletion of Tag-binding site II in pPX*dl*26/BV-1.

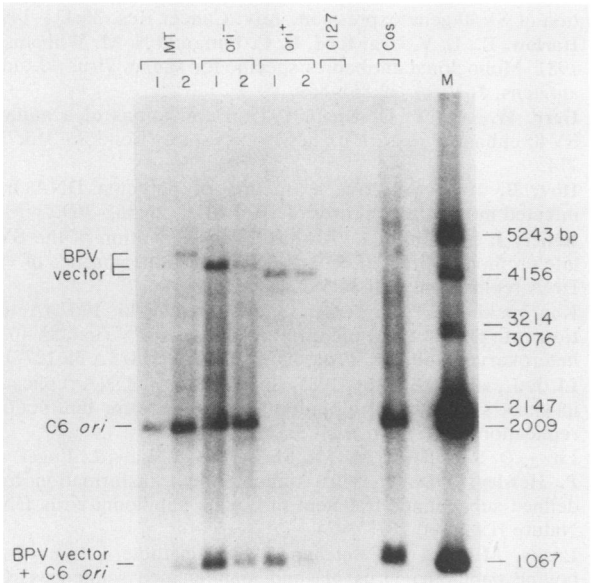

FIG. 4. Rescue and replication of the SV40 origin from C6 monkey cells. C6 monkey cells were cocultured with the indicated cell lines. Cultures were fused with polyethylene glycol and harvested by Hirt procedure after 48 h. DNA was digested with *Hpa*I and *Sal*I to generate a 2,009-bp fragment (indicated by C6 *ori*) specific for excision and replication of the SV40 genome in the C6 chromosome. Digests were run on a 1.4% agarose gel, blotted to nitrocellulose, and probed with nick-translated SV40 DNA. COS and C127 cells are shown as positive and negative controls, respectively. Lane M, Markers.

Even though mouse cells transformed with the SV40 Tag-BPV vectors are capable of expression of Tag levels equal to or greater than those synthesized in permissive COS1 cells, SV40 DNA is totally incapable of replication in these cells. We determined that the Tag synthesized in the transformed mouse cells was functional by two different assays. First, the ability to rescue origin sequences after fusion to C6 cells indicated that the Tag was functional when the permissive environment of a monkey cell was provided. Second, that purified mouse Tag can replicate SV40 DNA in vitro also suggests that it is fully functional. Neither of these results address the possibility that the mouse Tag is modified in vivo after fusion to C6 monkey cells or in vitro by incubation in the 293 cell extract. However, if this is the

case, then the modifying activity would be considered a permissivity factor which is absent from mouse cells.

Previous experiments (12) indicated that microinjection of large quantities of SV40 DNA and expression of large amounts of Tag in mouse cells will produce SV40 virions. One possible explanation for the difference between that study and our results may be that stably transformed cells expressing a high level of Tag are somehow different from untransformed mouse cells which only transiently express Tag. In addition to the high Tag concentration, microinjection results in a high concentration of DNA template which may shift the balance towards DNA replication. However, another possible explanation is that the phenomenon of replication-independent late gene activation (2, 16) is responsible for the production of late viral gene products and subsequent encapsidation of SV40 DNA into virions in mouse cells.

Development of the in vitro SV40 DNA replication system allowed us to check these hypotheses in greater detail; extracts prepared from mouse cells failed to support SV40 DNA replication in vitro, while extracts prepared from monkey or human cells were very active (18). Furthermore, it was demonstrated recently (23) that mouse DNA polymerase α-primase complex was unable to substitute for the human DNA polymerase α-primase complex in SV40 DNA replication in vitro. It would appear from these data that the primate enzyme complex is a permissivity factor. Nevertheless, two other sets of data would indicate that additional factors may also be involved in the phenomenon of permissivity. First, analysis of human fibroblasts transformed by the early region of SV40 DNA revealed that only 2 to 3% of the transformed cells synthesize free viral DNA (35), even though all cells contain both human DNA polymerase α-primase and wild-type Tag. The existence of additional factors may explain why replication of SV40 DNA occurs in only a small proportion of the cells. Second, infection of preimplantation mouse embryos with SV40 results in the synthesis of SV40 early and late proteins as well as the production of infectious SV40 progeny (1). Because these mouse experiments were performed by virus infection, it is possible to assume (although it has not been proven) that the virion synthesis was accomplished via the usual DNA replication and late protein synthesis pathway. This, in turn, would suggest that under certain specific conditions mouse DNA polymerase α-primase complex could support the replication of the SV40 DNA.

In summary, the inability of SV40 DNA to replicate in mouse cells is not affected by the level of synthesis of wild-type SV40 Tag. It is possible that the major factor responsible for replication of SV40 DNA in permissive cells is the primate DNA polymerase α-primase complex, but additional data indicate that other factors play a role in this phenomenon.

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LITERATURE CITED

1. **Abramczuk, J., A. Vorbrodt, D. Solter, and H. Koprowski.** 1978. Infection of mouse preimplantation embryos with simian virus 40 and polyoma virus. Proc. Natl. Acad. Sci. USA **75**:999–1003.
2. **Brady, J., J. B. Bolen, M. Radonovich, N. Salzman, and G. Khoury.** 1984. Stimulation of simian virus 40 late gene expression by simian virus 40 tumor antigen. Proc. Natl. Acad. Sci. USA **81**:2040–2044.
3. **Croce, C. M., and H. Koprowski.** 1973. Enucleation of cells made simple and rescue of SV40 by enucleated cells made even simpler. Virology **51**:227–229.
4. **DePamphilis, M. L., and P. M. Wassarman.** 1982. Organization and replication of papovavirus DNA, p. 37–114. *In* A. S. Kaplan (ed.), Organization and replication of viral DNA. CRC Press, Inc., Boca Raton, Fla.
5. **DiMaio, D., R. Treisman, and T. Maniatis.** 1982. Bovine papillomavirus vector that propagates as a plasmid in both mouse and bacterial cells. Proc. Natl. Acad. Sci. USA **79**:4030–4034.
6. **Gerard, R. D., and Y. Gluzman.** 1985. New host cell system for regulated simian virus 40 DNA replication. Mol. Cell. Biol. **5**:3231–3240.
7. **Gerber, P.** 1966. Studies on the transfer of subviral infectivity from SV40-induced hamster tumor cells to indicator cells. Virology **28**:501–509.
8. **Gluzman, Y.** 1981. SV40-transformed simian cells support the replication of early SV40 mutants. Cell **23**:175–182.
9. **Gluzman, Y., and B. Ahrens.** 1982. SV40 early mutants that are defective for viral DNA synthesis but competent for transformation of cultured rat and simian cells. Virology **123**:78–92.
10. **Gluzman, Y., J. Davison, M. Oren, and E. Winocour.** 1977. Properties of permissive monkey cells transformed by UV-irradiated simian virus 40. J. Virol. **22**:256–266.
11. **Graessman, A., M. Graessman, E. Guhl, and C. Mueller.** 1978. Quantitative correlation between simian virus 40 T-antigen synthesis and late viral gene expression in permissive and non-permissive cells. J. Cell Biol. **77**:R1–R8.
12. **Graessman, A., M. Graessmann, and C. Mueller.** 1981. Regulation of SV40 gene expression. Adv. Cancer Res. **35**:111–149.
13. **Harlow, E., L. V. Crawford, D. C. Pim, and N. M. Williamson.** 1981. Monoclonal antibodies specific for simian virus 40 tumor antigens. J. Virol. **39**:861–869.
14. **Herr, W., and Y. Gluzman.** 1985. Duplications of a mutated SV40 enhancer restore its activity. Nature (London) **313**:711–714.
15. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
16. **Keller, J. M., and J. C. Alwine.** 1984. Activation of the SV40 late promoter: direct effects of T antigen in the absence of viral DNA replication. Cell **36**:381–389.
17. **Koprowski, H., F. C. Jensen, and Z. Steplewski.** 1967. Activation of production of infectious tumor virus SV40 in heterokaryon cultures. Proc. Natl. Acad. Sci. USA **58**:127–133.
18. **Li, J. J., and T. J. Kelly.** 1985. Simian virus 40 DNA replication in vitro: specificity of initiation and evidence for bidirectional replication. Mol. Cell. Biol. **5**:1238–1246.
19. **Lowy, D. R., I. Dvoretsky, R. Shober, M.-F. Law, L. Engel, and P. Howley.** 1980. In vitro tumorigenic transformation by a defined subgenomic fragment of bovine papilloma virus DNA. Nature (London) **287**:72–74.
20. **Lusky, M., and M. Botchan.** 1984. Characterization of the bovine papilloma virus plasmid maintenance sequences. Cell **36**:391–401.
21. **Luthman, H., and G. Magnusson.** 1983. High efficiency polyoma DNA transfection of chloroquine treated cells. Nucleic Acids Res. **11**:1295–1308.
22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Murakami, Y,. C. R. Wobbe, L. Weissbach, F. B. Dean, and J. Hurwitz.** 1986. Role of DNA polymerase α and DNA primase in simian virus 40 DNA replication in vitro. Proc. Natl. Acad. Sci.

USA **83**:2869–2873.

24. **Poste, G., B Schaeffer, P. Reeve, and D. J. Alexander.** 1974. Rescue of simian virus 40 (SV40) from SV40-transformed cells by fusion with anucleate monkey cells and variation in yield of virus rescued by fusion with replicating or nonreplicating monkey cells. Virology **60**:85–95.
25. **Sambrook, J., L. Rodgers, J. White, and M.-J. Gething.** 1985. Lines of BPV-transformed murine cells that constitutively express influenza virus hemagglutinin. EMBO J. **4**:91–103.
26. **Sharma, S., L. Rodgers, J. Brandsma, M.-J. Gething, and J. Sambrook.** 1985. SV40 T antigen and the exocytotic pathway. EMBO J. **4**:1479–1489.
27. **Simanis, V., and D. P. Lane.** 1985. An immunoaffinity purification procedure for SV40 large T antigen. Virology **144**:88–100.
28. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by agarose gel electrophoresis. J. Mol. Biol. **98**:503–515.
29. **Stillman, B., R. D. Gerard, R. A. Guggenheimer, and Y. Gluzman.** 1985. T antigen and template requirements for SV40 DNA replication *in vitro*. EMBO J **4**:2933–2939.
30. **Stillman, B. W., and Y. Gluzman.** 1985. Replication and supercoiling of simian virus 40 DNA in cell extracts from human cells. J. Mol. Biol. **5**:2051–2060.
31. **Tegtmeyer, P.** 1972. Simian virus 40 deoxyribonucleic acid synthesis: the viral replicon. J. Virol. **10**:591–598.
32. **Tegtmeyer, P., M. Schwartz, J. K. Collins, and K. Rundell.** 1975. Regulation of tumor antigen synthesis by simian virus 40 gene *A*. J. Virol. **16**:168–178.
33. **Tooze, J. (ed.).** 1981. DNA tumor viruses: molecular biology of tumor viruses, 2nd ed., part 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Zinn, K., D. DiMaio, and T. Maniatis.** 1983. Identification of two distinct regulatory regions adjacent to the human β-interferon gene. Cell **34**:865–879.
35. **Zouzias, D., K. K. Jha, C. Mulder, C. Basilico, and H. L. Ozer.** 1980. Human fibroblasts transformed by the early region of SV40 DNA: analysis of "free" viral DNA sequences. Virology **104**:439–453.