MOLECULAR AND CELLULAR BIOLOGY, Nov. 1984, p. 2406–2412
0270-7306/84/112406-07$02.00/0

Vol. 4, No. 11

Isolation of Large T Antigen-Producing Mouse Cell Lines Capable of Supporting Replication of Polyomavirus-Plasmid Recombinants

WILLIAM J. MULLER, MONICA A. NAUJOKAS, AND JOHN A. HASSELL*

Department of Microbiology and Immunology, McGill University, Montreal, Quebec H3A 2B4, Canada

Received 2 July 1984/Accepted 21 August 1984

Construction of polyomavirus vectors, analysis of mutant viruses, and rescue of integrated polyomavirus genomes would be considerably aided by the availability of transformed, permissive mouse cell lines capable of producing the viral tumor antigens. To isolate such cell lines, we constructed a hybrid transcription unit composed of the simian virus 40 early promoter fused to the coding region for the polyomavirus tumor antigens. This hybrid transcription unit was used to transform NIH 3T3 cells. Independent foci of transformed cells were isolated, recloned, and characterized. Among 10 lines initially analyzed, 7 supported the replication of origin-bearing plasmid DNAs. Three cell lines were characterized in greater detail. Each line contained one or two independent insertions of polyomavirus DNA and synthesized all three viral tumor antigens. Moreover, the large tumor antigen in two of three lines bound with specificity to sequences about the polyomavirus origin and early promoter. These cell lines should prove useful for studying not only the replication of polyomavirus but also the expression of foreign genes in a mouse cell environment.

Polyomavirus has served historically as a model to study mammalian gene expression, DNA replication, and tumorigenesis (12). Moreover, now that the viral DNA molecule has been completely sequenced (7, 33), the viral mRNAs have been mapped (37), and the viral proteins have been identified (17), interest in the use of the virus as a vector to introduce and study the expression of foreign genes in murine hosts has developed (14, 16). The development of such vectors has been hampered in part by a lack of detailed knowledge of the borders of viral regulatory sequences (i.e., early and late promoters and the origin for DNA replication) and by the absence of permissive cell lines capable of supporting the replication of defective viruses. The availability of such cell lines would obviate the necessity of using helper viruses to complement stocks of defective viruses bearing foreign genes, and consequently, pure populations of helper-free, defective viruses could be produced. In addition, such complementing permissive cells would also prove useful in mapping some of the viral regulatory elements required for replication and therefore aid in the construction of polyomavirus vectors.

Numerous virus-host systems have been described in which permissive cells have been used to complement the replication of mutant viruses. For example, COS cells, permissive monkey cells transformed by an origin-defective mutant of simian virus 40 (SV40), have been isolated which support the replication of early SV40 mutants (10). There are also permissive cell lines transformed by adenoviruses (11, 13, 32), herpesviruses (22), and Moloney murine leukemia virus (19) which can serve as hosts for mutant viruses or virus vectors. These cell lines generally express viral proteins from integrated viral DNA whose genes are either missing from the virus vector or are altered by mutation in the mutant viruses. Many of these cell lines have also proved useful to study the transient expression of foreign genes linked to a viral replicon and therefore replicated to high copy number in the transfected cells (6).

* Corresponding author.

Our initial objective in isolating permissive mouse cells capable of complementing the replication of mutant polyomaviruses was to use such cells to map the borders of the polyomavirus functional origin for DNA replication (24). To isolate permissive mouse cell lines which synthesize the polyomavirus early proteins, we transformed NIH 3T3 cells with a hybrid transcription unit composed of the SV40 early promoter fused to the early region of polyomavirus. Most of the resulting transformed mouse cells supported the replication of recombinant plasmids which contained the polyomavirus origin for DNA replication but not the sequences encoding large T antigen. Therefore, these transformed permissive mouse cells are capable of providing functional polyomavirus large T antigen in *trans*. They should prove useful to map the borders of the virus origin for DNA replication, to isolate mutants with lesions in the viral T antigens, to rescue integrated polyomavirus genomes from transformed cells, to study the transient expression of foreign genes cloned in polyomavirus vectors, and as hosts for the replication of helper-free recombinant viruses.

MATERIALS AND METHODS

Cell culture. All the cell lines used in this study were propagated as monolayer cultures in Dulbecco modified Eagle medium (DMEM) containing 10% calf serum and gentamicin (50 μg/ml). The cultures were maintained at 37°C in a humidified-CO_2 atmosphere. Cells were routinely passaged after they reached confluence by trypsinization and replated at ca. 10^4 cells per cm^2 of surface area.

Preparation of plasmid DNA and its modification. Recombinant plasmid DNAs were isolated from bacteria and purified by CsCl density gradient centrifugation. Digestions of these DNAs with restriction endonucleases were performed in accordance with the conditions specified by their manufacturers.

Reaction of DNA with the Klenow fragment of DNA polymerase I was performed by incubating 1 μg of DNA in 50 μl of 10 mM Tris hydrochloride (pH 7.6), 5 mM $MgCl_2$, 1 mM dithiothreitol, the appropriate deoxynucleotide triphosphates (0.1 to 1.0 mM), and 5 U of the Klenow fragment of

Escherichia coli DNA polymerase I for 1 h at 15°C. The reaction was terminated by sequential phenol and chloroform-isoamyl alcohol (24:1, vol/vol) extraction, and the DNA was isolated by ethanol precipitation. Ligation of DNA with T4 ligase was carried out as described previously (23).

Construction of recombinant plasmids. The construction and final structure of pPH1-8 (15), pdPB1a and pdPR1a (25), and pPSVE1 (M. S. Featherstone, M. A. Naujokas, B. J. Pomerantz, and J. A. Hassell, Nucleic Acids Res., in press) have been described previously. pPSVE1-B1a (see Fig. 1) was constructed by ligating the carboxy-terminal coding sequences for large T antigen, the *Eco*RI-to-*Bam*HI (nucleotides 1560 to 4632) (33) DNA fragment from pdBP1a DNA (a genomic clone of polyomavirus cloned as a *Bam*HI fragment in pML2 DNA), to *Eco*RI-cleaved pPSVE1 DNA. pPSVE1 comprises pBR322 DNA and the SV40 early promoter (the SV40 *Hin*dIII C fragment) fused to the coding sequences for small and middle T antigen (polyomavirus nucleotides 154 to 1560). After ligation of the *Eco*RI-terminated ends with T4 ligase, the linear molecules were treated with the Klenow fragment of DNA polymerase I from *E. coli* in the presence of all four deoxynucleotide triphosphates to fill in the single-stranded ends and the molecules circularized by reaction with T4 ligase (29). The reaction mixture was used to transform *E. coli* HB101 to ampicillin resistance, and individual colonies were isolated. Several transformed colonies were screened by restriction endonuclease analysis for the desired recombinant plasmid. After these colonies were found, one was chosen for further study. The structure of the plasmid DNA carried by these bacteria was confirmed by digestion with many restriction endonucleases (see Fig. 1).

Isolation of MOP cell lines. The mouse originless polyomavirus (MOP) cell lines were established by transforming NIH 3T3 cells with pPSVE1-B1a DNA. In brief, 10 separate cultures of sparsely seeded NIH 3T3 cells (1×10^5 to 2×10^5 cells per 100-mm diameter petri dish) were transfected with CsCl-purified, supercoiled pPSVE1-B1a DNA (100 ng per petri dish) by the calcium phosphate technique (41). The calcium phosphate-containing medium was removed 5 h after its initial application, and fresh DMEM containing serum was added. The medium was then changed every 3 days until foci of transformed cells appeared; 50 to 100 foci appeared per petri dish after 2 weeks, and one focus was isolated from each dish and subsequently cloned (15). A total of 10 independent colonies were established and characterized.

DNA replication assay. CsCl gradient-purified, supercoiled plasmid DNAs were transfected into cells by a modification of the DEAE-dextran transfection technique (20, 34). A 60-mm dish containing ca. 5×10^5 cells was washed with 5 ml of serum-free DMEM, and the cells were then incubated with 1 ml of DMEM supplemented with 250 μg of DEAE-dextran (molecular weight, 500,000) per ml and 1 μg of plasmid DNA at 37°C for 4 h in a humidified CO_2 incubator. The cells were then washed twice with 5 ml of serum-free DMEM and maintained in 5 ml of DMEM containing 10% calf serum.

At 72 h posttransfection, low-molecular-weight plasmid DNA was isolated from the cells by the Hirt extraction procedure (18). After sedimentation of high-molecular-weight DNA, 0.5 ml of the cleared lysate was diluted in 4.5 ml of TE buffer (10 mM Tris hydrochloride, pH 8.0, 1 mM EDTA) and extracted once with buffer-saturated phenol and once with chloroform-isoamyl alcohol (24:1). Nucleic acids were precipitated by the addition of 2.5 volumes of cold ethanol and were stored overnight at −20°C. Precipitates were collected by centrifugation and suspended in 50 μl of TE buffer. A 15-μl portion of the DNA sample was sequentially digested with *Bam*HI and *Dpn*I restriction endonucleases.

The digested plasmid DNAs were subjected to electrophoresis through 1.0% (wt/vol) agarose gels, and the DNA fragments were transferred to nitrocellulose filters by the Southern technique (35). The nitrocellulose filters were hybridized to ^{32}P-labeled, nick-translated pdPR1a DNA (10^8 cpm/μg) by using dextran sulfate (40). After the filters were washed, they were dried and autoradiographed for 3 to 12 h with Kodak XAR-5 film and DuPont Lightning-Plus intensifying screens.

DNA and protein analyses. The methods used to isolate

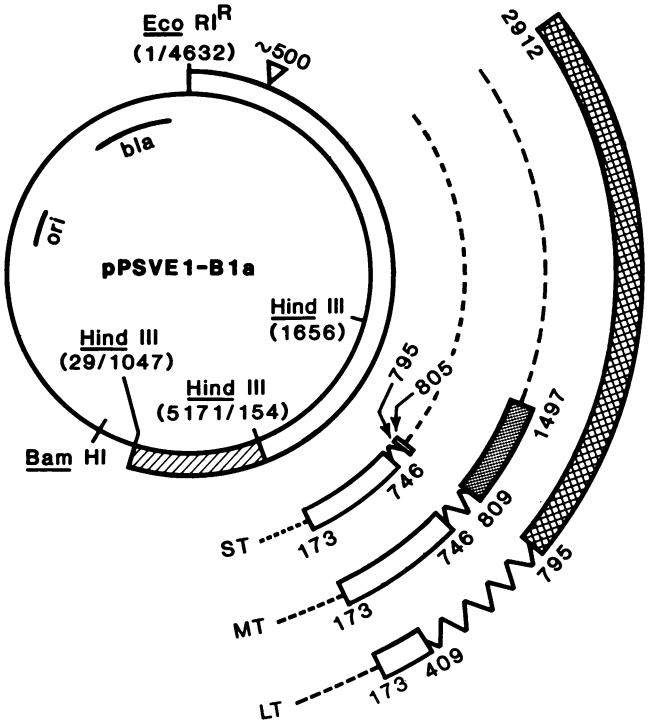

FIG. 1. Structure of pPSVE1-B1a DNA. pPSVE1-B1a DNA (ca. 9,430 base pairs [bp]) comprises pBR322 DNA (4,333 bp; thin line), the *Hin*dIII C fragment of SV40 DNA (1,119 bp; hatched box), and polyomavirus DNA (ca, 3,978 bp; open box). The sequences in pBR322 DNA between its *Eco*RI (nucleotide 1) and *Hin*dIII (nucleotide 29) sites were substituted with the SV40-polyomavirus hybrid transcription unit. The pBR322 *Eco*RI (nucleotide 1) and the polyomavirus *Bam*HI (nucleotide 4632) sites were destroyed at one joint, but the *Hin*dIII site was preserved at the other joint. The SV40 sequences are arranged such that its early promoter drives transcription of the polyomavirus early region. The polyomavirus sequences contain a deletion of about 500 bp (denoted by the triangle) encompassing the *Hin*dIII site at nucleotide 3918. The nucleotide schemes of Sutcliffe (36), Buchman et al. (4), and Soeda et al. (33) were used for pBR322, SV40, and polyomavirus DNA, respectively. The structures of the polyomavirus early mRNAs (small [ST], middle [MT], and large [LT]) are illustrated outside the circular map of pPSVE1-B1a DNA. Transcription of the three coterminal mRNAs should originate in SV40 DNA and terminate in polyomavirus DNA. The boxed areas of each mRNA represent translated sequences, and their different shadings denote different reading frames. The jagged lines connecting the mRNA coding regions represent sequences which are spliced from the nuclear precursors of the various mRNAs. The splice sites were taken from Treisman et al. (37).

low- and high-molecular-weight cellular DNA and to characterize that DNA after restriction endonuclease cleavage and Southern blotting-hybridization have been described previously (31).

Immunoprecipitation of the viral T antigens and their identification by fluorography after electrophoresis through acrylamide-sodium dodecyl sulfate gels were also performed as described previously (31).

Binding of large T antigen to DNA. The preparation of the reagents and the methods used to measure the binding of large T antigen to end-labeled DNA fragments have all been described previously (30). The substrates used in the binding reaction were the *Hin*fI fragments of pPH1-8 DNA. This recombinant plasmid contains the origin-bearing *Hin*dIII-1 fragment of polyomavirus DNA cloned within the *Hin*dIII site of pBR322 (15). The *Hin*fI-4 fragment of pPH1-8 DNA (polyomavirus nucleotides 5073 to 385) contains all the large T antigen-binding sites (30).

RESULTS

Transformation of NIH 3T3 cells with DNA containing a hybrid transcription unit. Isolation of permissive mouse cells which express functional polyomavirus large T antigen requires the use of a mutant viral DNA molecule with a defective origin. This is so because origin-containing DNAs are not stably maintained in the integrated state in the genome of a permissive cell producing functional large T antigen (28). This problem can be avoided by using an origin-defective mutant DNA for transformation or by replacing the entire polyomavirus early promoter and origin region with a foreign promoter. We chose the latter approach because the exact borders of the polyomavirus origin had not been defined when we began these experiments, thereby making it difficult to choose an appropriate site for mutagenesis. Moreover, the precise borders and the important controlling elements of the early promoter also had not been defined, and therefore we had no way of knowing whether an origin-defective molecule would also prove to be debilitated in expression. Therefore, we chose to replace the entire upstream noncoding region of polyomavirus with that of another strong promoter.

The SV40 early promoter has been well characterized (1, 8), and we have shown that it functions nearly as well as the early polyomavirus promoter to drive expression of downstream genes in rodent cells (Featherstone et al., in press). The *Hin*dIII C fragment of SV40 contains all the viral regulatory elements, including the origin for DNA replication as well as the early and late promoters and the early start sites for transcription (1, 2, 8). Therefore, we placed this SV40 DNA fragment in the appropriate orientation before the early region of polyomavirus.

During lytic infection of mouse cells by polyomavirus, expression of the T antigens is repressed at late times by the binding of large T antigen to promoter-proximal DNA sequences, thereby retarding transcription initiation (5). The binding sites for polyomavirus large T antigen all lie upstream of nucleotide position 154 in polyomavirus DNA (30). Moreover, the polyomavirus principal early mRNA 5′ termini also map upstream of nucleotide position 154 (they are located between nucleotide positions 147 and 153) (18, 37). Because we wished to maximize expression of the polyomavirus T antigens in permissive mouse cells, we attempted to ensure against repression of early transcription. Therefore, we fused the SV40 early promoter to the polyomavirus early region beginning at nucleotide position 154 in polyomavirus DNA. In this way we isolated a hybrid transcription unit, pPSVE1-B1a, whose coding sequences are expressed from a strong promoter and are independent of regulation (Fig. 1). We expected that pPSVE1-B1a would be transcribed in mouse cells to yield hybrid mRNA molecules whose 5′ untranslated termini were derived from SV40 DNA but whose translated and 3′ noncoding sequences were derived from polyomavirus DNA.

pPSVE1-B1a DNA was used to transfect untransformed NIH 3T3 cells to the transformed phenotype as described previously. Transformed foci appeared in the cultures after 2 weeks. Ten independent foci were isolated from separate plates and recloned once before they were analyzed further. We named these lines MOP cells by analogy to COS cells (CV-1 originless SV40) (10).

Replication of origin-bearing plasmid DNA in MOP cell lines. The MOP cell lines need only express middle T antigen to maintain the transformed cell phenotype (15, 23, 38). However, the coding sequences for middle and large T antigen overlap at least over a part of the viral genome (Fig. 1), and therefore we expected that a fair proportion of the lines would gratuitously produce large T antigen. To determine whether this was true and to ensure that active large T antigen was synthesized, we screened the 10 MOP lines for their capacity to support the replication of a recombinant plasmid bearing the polyomavirus functional origin for DNA replication.

We chose the plasmid pdPR1A for this analysis (25). pdPR1A comprises pML2 DNA and the entire polyomavirus genome. The viral DNA was inserted at the *Eco*RI site

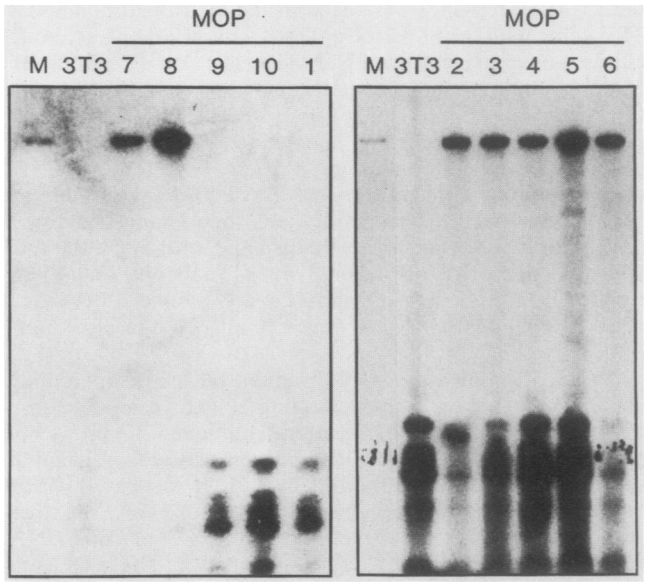

FIG. 2. Replication of pdPR1a DNA in the various MOP cell lines. A 1-μg sample of pdPR1a was transfected into ca. 5×10^5 cells growing on the surface of a plastic petri dish (6-cm diameter) by using DEAE-dextran as a facilitator of DNA uptake. Replication of the pdPR1a DNA was measured by isolating low-molecular-weight DNA and cleaving it with *Dpn*I and *Sal*I as described in the text. There are many sites of cleavage for *Dpn*I, but only one *Sal*I cleavage site in pdPR1a DNA. The replicated DNA is resistant to *Dpn*I cleavage and appears as a band which comigrates with a linear marker of pdPR1a DNA (M). The marker lane (M) contained 10^{-5} μg of pdPR1a DNA. The input unreplicated DNA appears as a smear of low-molecular-weight fragments at the bottom of the autoradiogram.

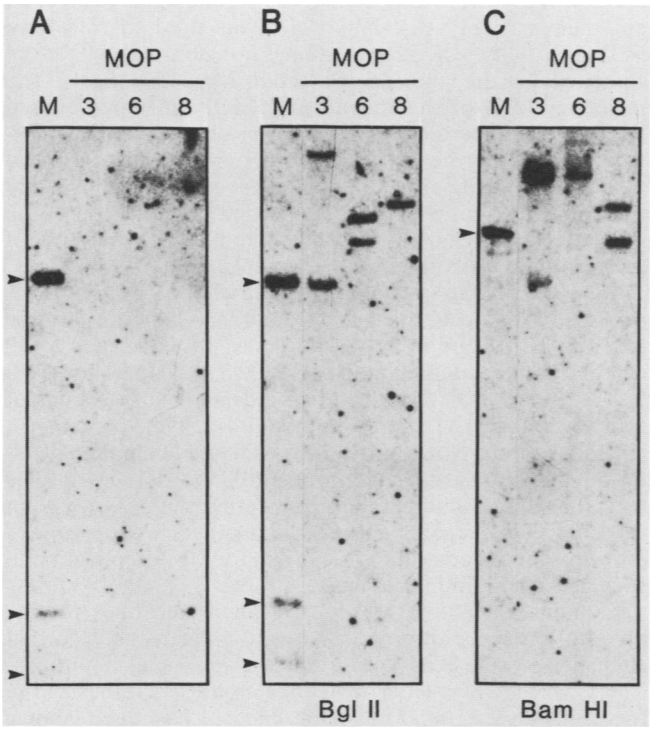

FIG. 3. Characterization of MOP cellular DNA. (A) Low-molecular-weight DNA was isolated from three MOP cell lines and probed for the presence of pPSVE1-B1a sequences by Southern blotting-hybridization as described in the text. The lane marked M in (A) contained a *Hin*dIII digest of 10^{-5} μg of pPSVE1-B1a DNA. Three fragments of ca. 6,809, 1,502, and 1,119 base pairs are displayed on the autoradiogram. (B) High-molecular-weight cellular DNA was isolated from three MOP cell lines and digested with *Bgl*II, which does not cleave the transforming pPSVE1-B1a DNA. The fragments complementary to pPSVE1-B1a DNA were detected by Southern blotting-hybridization and autoradiography as described in the text. The lane marked M contained a *Hin*dIII digest of 10^{-5} μg of pPSVE1-B1a DNA. (C) High-molecular-weight cellular DNA from three MOP lines was hydrolyzed with *Bam*HI, which cleaves the transforming DNA, pPSVE1-B1a, once. The fragments bearing sequences homologous to pPSVE1-B1a DNA were detected as described previously. The lane labeled M contained 10^{-5} μg of *Bam*HI-cleaved pPSVE1-B1a DNA. Arrows indicate the positions of the marker fragments.

of PML2 as an *Eco*RI genomic fragment. Because *Eco*RI cleaves polyomavirus DNA in the middle of the early region (at nucleotide number 1560) (Fig. 1), this plasmid cannot encode large T antigen. Therefore, pdPR1a will only replicate in permissive cells which produce active large T antigen in *trans*.

The replicative capacity of pdPR1a DNA was assessed by transfection of the MOP cells, extraction of low-molecular-weight DNA 72 h later, and measurement of the state of methylation of the recombinant DNA as an indirect assay of replication by digestion with *Dpn*I and *Sal*I followed by Southern blotting-hybridization (24, 25, 27). This assay takes advantage of the fact that plasmid DNA isolated from *E. coli* is methylated at adenine residues which lie within the sequence GATC. Such methylated sequences are recognized and cleaved by *Dpn*I. DNA isolated from bacteria and subsequently replicated in mammalian cells is not methylated and therefore becomes resistant to *Dpn*I. To collect all the *Dpn*I-resistant, replicated plasmid DNA in a single band after gel electrophoresis, we digested this DNA with *Sal*I, which cleaves pdPR1a once. After electrophoresis and Southern blotting-hybridization, it is possible to easily distinguish the replicated DNA from the input, *Dpn*I-sensitive unreplicated DNA (27). The replicated DNA appears as a single species migrating with a linear marker of pdPR1a DNA, whereas the unreplicated DNA appears as numerous fragments of poorly separated, low-molecular-weight DNA at the bottom of the autoradiogram.

The outcome of screening 10 MOP cell lines for their capacity to support pdPR1a replication is illustrated in Fig. 2. Seven of the 10 lines supported pdPR1a DNA replication to approximately the same extent (Fig. 2). As expected, the parental cell line (NIH 3T3) did not support pdPR1a replication because this line does not synthesize polyomavirus large T antigen. These results show that many of the transformed MOP cell lines express active large T antigen. Because there were no large differences in the capacities of the various lines to complement polyomavirus DNA replication, we randomly chose three lines, MOP-3, -6, and -8, for further characterization.

MOP cells with integrated pPSVE1-B1a DNA. To determine the physical state of pPSVE1-B1a DNA in the MOP-3, -6, and -8 cell lines, we screened these cells for the presence of free plasmid DNA by preparing low-molecular-weight DNA from them and assaying this DNA for the presence of sequences homologous to pPSVE1-B1a DNA. We could not detect free plasmid DNA in these cells (Fig. 3A). Because we assayed the DNA from 3×10^6 cells and because we could readily detect 10^{-5} μg of marker DNA, we estimate that there are fewer than 0.3 molecules of free pPSVE1-B1a DNA per cell.

Digestion of the MOP cell DNA with a restriction endonuclease which does not cleave pPSVE1-B1a DNA would establish whether this DNA is integrated, and the number of viral fragments detected after Southern blotting-hybridization would establish the number of separate insertions of the transforming DNA (3). *Bgl*II is an enzyme which does not cleave pPSVE1-B1a DNA. Digestion of MOP-3, -6, and -8 cellular DNA with *Bgl*II followed by agarose gel electrophoresis and Southern blotting-hybridization revealed the presence of integrated pPSVE1-B1a DNA in each of the cell lines (Fig. 3B). MOP-3 and MOP-6 cells contained two insertions, whereas MOP-8 cells contained a single insertion of transforming DNA.

To determine whether any of the three MOP cell lines contained multiple, tandemly reiterated pPSVE1-B1a genomes arranged in a head-to-tail fashion, we cleaved their cellular DNAs with *Bam*HI, which cleaves pPSVE1-B1a DNA once. If the *Bam*HI site was tandemly repeated, then we would expect to release linear pPSVE1-B1a DNA after cleavage of cellular DNA with *Bam*HI. None of the three MOP cell lines yielded unit-length pPSVE1-B1a DNA after cleavage with *Bam*HI (Fig. 3C). The cellular DNA of the MOP-8 line yielded two fragments after cleavage with *Bam*HI, which is consistent with a single insertion of pPSVE1-B1a in this cell line and the retention of the *Bam*HI site within the integrated DNA. These results show that each of the MOP cell lines examined contained a small number of integrated pPSVE1-B1a genomes (from one to two) and that these insertions were not made up of long arrays of tandemly reiterated pPSVE1-B1a DNA.

Viral T antigens expressed by MOP cell lines. We examined the three prototype MOP cell lines for their capacity to express the polyomavirus T antigens. This was accomplished by immunoprecipitation of ^{35}S-labeled cell lysates

with serum from tumor-bearing rats (Fig. 4). The three MOP cell lines contained large, middle, and small T antigen although it was difficult to invariably demonstrate the presence of small T antigen in the MOP-6 and MOP-8 cell lines (Fig. 4). In separate, independent experiments we could demonstrate no large differences in the amount of large, middle, or small T antigen synthesized by the various cell lines (data not shown).

One biochemical activity associated with polyomavirus large T antigen is its capacity to bind to specific viral sequences near the origin for DNA replication and the early promoter (9, 29). To determine whether the large T antigen produced by the MOP cell lines could bind to polyomavirus DNA with specificity, we used the indirect assay described by McKay (21). Terminally labeled *Hin*fI fragments of pPH1-8 DNA were incubated with crude nuclear extracts from lytically infected 3T6 cells, MOP-3 cells, and MOP-8 cells, and the fragments bearing bound large T antigen were immunoprecipitated with antitumor serum and *Staphylococcus aureus* bacteria. The immunoprecipitated DNAs were separated by agarose gel electrophoresis and visualized by autoradiography (Fig. 5). The large T antigen from each cell line specifically bound to the *Hin*f-4 fragment of pPH1-8 DNA, which carries all of the binding sites (30). Surprisingly, the amount of nuclear extract required to immunoprecipitate an equivalent amount of the *Hin*fI-4 fragment was nearly the same for all three extracts. These results show that all three MOP cell lines analyzed contained large T antigen as well as middle and small T antigen and that two of these lines (MOP-3 and MOP-8) contained large T antigen molecules capable of binding to the same viral sequences as those recognized by the large T antigen synthesized in lytically infected 3T6 cells.

DISCUSSION

We constructed a recombinant plasmid, pPSVE1-B1a, containing a hybrid transcription unit comprising the SV40 early promoter fused to the T antigen coding sequences of polyomavirus. pPSVE1-B1a DNA was used to transform NIH 3T3 cells, and the resulting transformed cells were screened for the presence of active large T antigen. The majority (70%) of the transformed MOP cell lines proved capable of supporting the replication of recombinant plasmids containing the polyomavirus origin for DNA replication. These results prove that permissive mouse cells readily tolerate active large T antigen provided that excision of the integrated viral DNA is blocked by removal or mutation of the polyomavirus origin for DNA replication.

We analyzed the state and structure of pPSVE1-B1a DNA in three MOP cell lines and found no evidence of free pPSVE1-B1a DNA in any of the lines. Instead, each MOP cell line contained integrated pPSVE1-B1a DNA. The MOP-3 and MOP-6 lines contained two independent insertions of transforming DNA, whereas the MOP-8 cell line contained a single insertion. None of the lines contained complete head-to-tail, tandemly arranged copies of pPSVE1-B1a DNA. The fact that free, unintegrated transforming DNA was not detected in these cells suggests that polyomavirus large T antigen cannot act at the SV40 origin for DNA replication to trigger excision in mouse cells.

Examination of the MOP-3, -6, an -8 cell lines for the physical presence of polyomavirus T antigens showed that each line synthesized large, middle, and small T antigen. These proteins were the same size as those synthesized in the transformed rat T1A1 cell line. It has been shown previously that the T1A1 T antigens are the same size as those synthesized in lytically infected mouse cells (16). It is very likely that the middle T antigen of the MOP cell lines is functional, because each line is transformed, and this phenotype is strongly correlated with middle T antigen activity (15, 23, 38). We do not know whether the small T antigen present in the MOP lines is active or not. However, the large T antigens were functional for DNA replication, and they were capable of specifically binding to the large T antigen binding

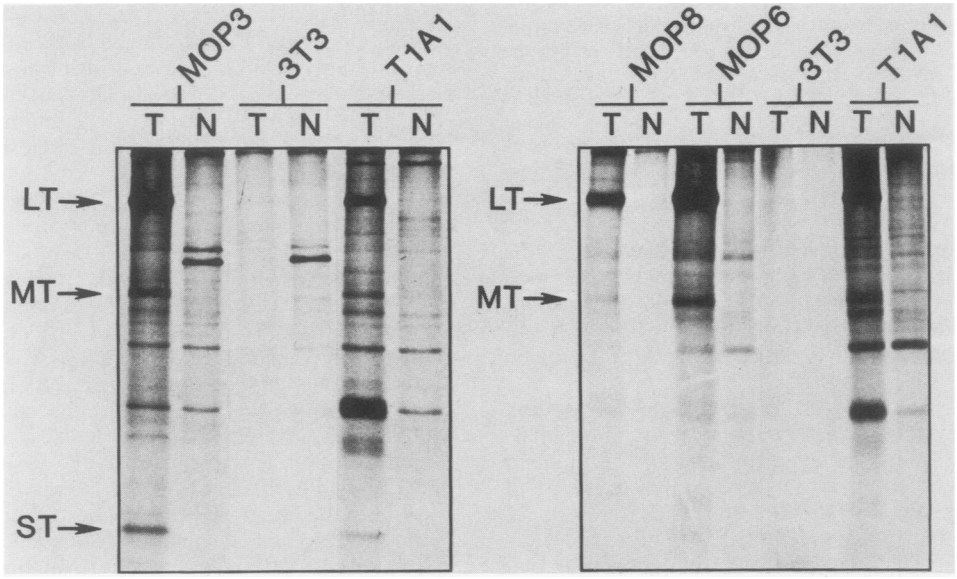

FIG. 4. Immunoprecipitation of [^{35}S]methionine-labeled T antigens from the MOP cell lines. ^{35}S-labeled proteins were immunoprecipitated from three MOP cell lines, NIH 3T3, and the T1A1 rat cell line with antiserum from a Fisher rat which bore a tumor induced by a polyomavirus-transformed rat cell line (T). Serum from a normal Fisher rat was used as a control (N). The immunoprecipitated proteins were separated by electrophoresis through a 12.5% sodium dodecyl sulfate-polyacrylamide gel and autoradiographed as described in the text. Each panel represents a separate gel. LT, MT, and ST refer to large, middle, and small T antigen, respectively. Small T antigen was not visible in the autoradiogram of the gel represented by the rightmost panel. However, this protein was detected on longer exposure of the gel and in separate experiments (data not shown).

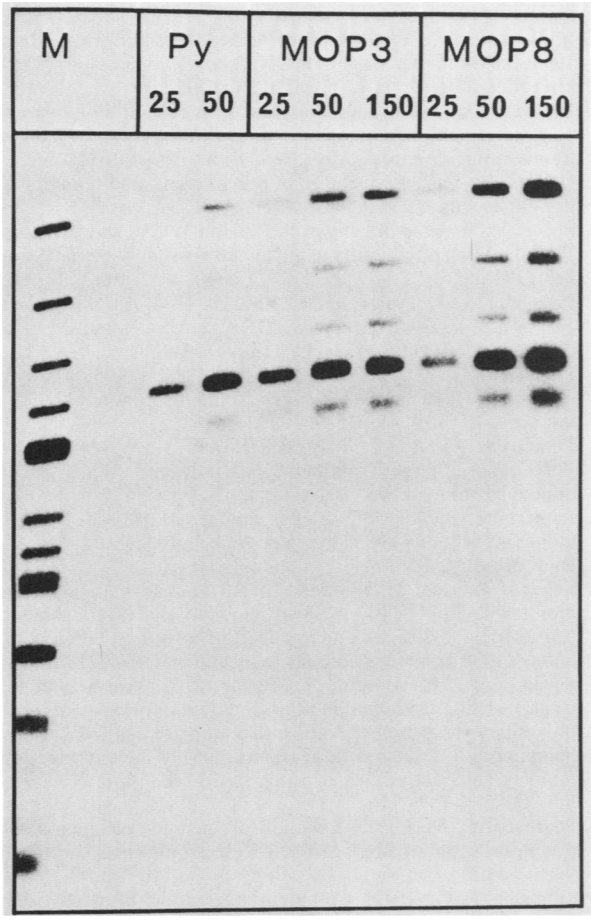

FIG. 5. Immunoprecipitation of ^{32}P end-labeled *Hin*fI fragments of pPH1-8 DNA after reaction with nuclear extract from various cell lines. Nuclear extracts were prepared from polyomavirus-infected 3T6 cells (Py), MOP-3 cells, and MOP-8 cells and were incubated with the end-labeled fragments of pPH1-8 DNA. Several volumes of nuclear extract, 25, 50, and 150 μl, were used. The lane marked M contained a portion of the original substrate (pPH1-8 cleaved with *Hin*fI) used in the binding reactions. The fourth-largest, 604-base-pair fragment contained the binding sites for large T antigen.

sites near the polyomavirus early promoter and origin for DNA replication.

We compared the extent to which the three MOP cell lines support the replication of origin-bearing plasmids and found no large differences among them. We estimate that each cell in the population yields from 500 to 2,000 replicated plasmid molecules by 72 h posttransfection (24, 25). Because less than 10% of the cell population was effectively transfected by this procedure, the yield of replicated DNA per transfected cell is at least 10 times higher than this.

We have also compared the yield of replicated plasmid DNA obtained from MOP-8 cells with that obtained from COS cells. These experiments were conducted by transfection of MOP-8 cells with pML2 containing the minimal polyomavirus origin for DNA replication [pdPP1(B)Bg(H); see reference 24] and by transfection of COS-1 cells with pML2 containing the *Hin*dIII C fragment of SV40. Both cell lines yielded the same number of replicated plasmid DNA molecules 72 h after transfection (E. Bennett and J. A. Hassell, manuscript in preparation).

An important application of transformed, permissive cells is their use to complement the replication of early mutants of polyomavirus, or early-replacement viral vectors. Unfortunately, the MOP cell lines which we characterized in greatest detail did not yield high titers of mutant viruses (data not shown). We believe that this was due to the nature of the cell line (NIH 3T3) rather than to the efficiency of complementation by large T antigen. Although 3T3 cells are permissive for polyomavirus replication, the yield of infectious virus produced by them is at least 1,000-fold reduced compared to infected 3T6 or baby mouse kidney cells (data not shown). To correct this deficiency, we have recently isolated another suite of transformed permissive 3T6 cells by using a slightly different transformation strategy. Among those lines are several which encode a temperature-sensitive large T antigen. We anticipate that these 3T6 MOP cell lines will support the efficient replication of early polyomavirus mutants and early-replacement vectors.

Two other transformed mouse cell lines have been described which support the replication of polyomavirus-plasmid recombinants (39). These lines were derived by transformation of C127 mouse cells with an origin-defective polyomavirus genome. Because a detailed characterization of these cell lines has not been published we cannot compare them to the MOP cell lines described here. Moreover, we also do not know whether C127 mouse cells permit the efficient replication of polyomavirus, and therefore we do not know whether their transformed derivatives allow for the production of defective polyomaviruses.

Despite this shortcoming, the MOP cell lines have already proved useful to map the borders of the polyomavirus origin for DNA replication (24), and they have other applications as well. For example, they can be used as a ready source of large T antigen for DNA binding studies or as a source of middle and small T antigen. The amount of active large T antigen we obtained from the MOP-6 and MOP-8 cell lines was nearly 30% that obtained from lytically infected 3T6 cells. The MOP cell lines can also be used to rescue integrated polyomavirus DNA sequences from transformed cells. Finally, by cloning foreign genes in pML2 plasmids bearing the polyomavirus origin, it should be possible to study their expression in mouse cells. Therefore, MOP cells provide an alternative to COS cells when it is important to measure gene activity in a murine cell environment.

ACKNOWLEDGMENTS

We thank Betsy Pomerantz for measuring the binding of large T antigen to DNA and Cathy Collins for excellent technical assistance.

This research was supported by funds from the Medical Research Council and the National Cancer Institute of Canada. W.J.M. is supported by a research studentship from the MRC and J.A.H. is a research associate of the NCI.

LITERATURE CITED

1. **Benoist, C., and P. Chambon.** 1981. *In vivo* sequence requirements of the SV40 early promoter region. Nature (London) **290**:304–309.
2. **Bergsma, D. J., D. M. Olive, S. W. Hartzell, and K. N. Subramanian.** 1982. Territorial limits and functional anatomy of simian virus 40 replication origin. Proc. Natl. Acad. Sci. U.S.A. **79**:381–385.
3. **Botchan, M., W. Topp, and J. Sambrook.** 1976. The arrangement of simian virus 40 sequences in DNA of transformed cells. Cell **6**:269–287.
4. **Buchman, A. R., L. Burnett, and P. Berg.** 1980. The SV40 nucleotide sequence, p. 799–823. *In* J. Tooze (ed.), DNA tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring

Harbor, N.Y.
5. **Cogen, B.** 1978. Virus specific early RNA in 3T6 cells infected by a tsA mutant of polyoma virus. Virology **85**:222–230.
6. **Elder, J. T., R. A. Spritz, and S. M. Weissman.** 1981. Simian virus 40 as a eukaryotic cloning vehicle. Annu. Rev. Genet. **15**:295–340.
7. **Friedmann, T., A. Esty, P. Laporte, and P. Deininger.** 1979. The nucleotide sequence and genome organization of the polyoma early region: extensive nucleotide and amino acid homology with SV40. Cell **17**:715–724.
8. **Fromm, M., and P. Berg.** 1982. Deletion mapping of DNA regions required for SV40 early region promoter function *in vivo*. J. Mol. Appl. Genet. **1**:457–481.
9. **Gaudray, P., D. Tyndall, R. Kamen, and F. Cuzin.** 1981. The high affinity binding site on polyoma virus DNA for the viral target protein. Nucleic Acids Res. **9**:5697–5710.
10. **Gluzman, Y.** 1981. SV40-transformed simian cells support the replication of early SV40 mutants. Cell **23**:175–182.
11. **Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36**:59–72.
12. **Griffin, B. E.** 1982. Structure and genomic organization of SV40 and polyoma virus, p. 61–123. *In* J. Tooze (ed.), DNA tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Grodzicker, T., and D. F. Klessig.** 1980. Expression of unselected adenovirus genes in human cells co-transformed with the HSV-1 tk gene and adenovirus 2 DNA. Cell **21**:458–463.
14. **Hassell, J. A., C. Mueller, A.-M. Mes, M. Featherstone, M. Naujokas, B. Pomerantz, and W. Muller.** 1982. The construction of polyoma virus vectors: functions required for gene expression, p. 71–77. *In* Y. Gluzman (ed.), Eukaryotic viral vectors. Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.
15. **Hassell, J. A., W. C. Topp, D. B. Rifkin, and P. Moreau.** 1980. Transformation of rat embryo fibroblasts by cloned polyoma virus DNA fragments containing only part of the early region. Proc. Natl. Acad. Sci. U.S.A. **77**:3978–3982.
16. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
17. **Ito, Y., and N. Spurr.** 1979. Polyoma virus T antigens expressed in transformed cells: significance of middle T antigen in transformation. Cold Spring Harbor Symp. Quant. Biol. **44**:149–157.
18. **Kamen, R., J. Favalaro, J. Parker, R. Treisman, L. Lania, M. Fried, and A. Mellor.** 1980. Comparison of polyoma virus transcription in productively infected mouse cells and transformed rodent cell lines. Cold Spring Harbor Symp. Quant. Biol. **44**:637–651.
19. **Mann, R., R. C. Mulligan, and D. Baltimore.** 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell **33**:153–159.
20. **McCutchan, T., and J. S. Pagano.** 1968. Enhancement of infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. J. Natl. Cancer Inst. **41**:351–357.
21. **McKay, R. D. G.** 1981. Binding of simian virus 40 T antigen-related protein to DNA. J. Mol. Biol. **145**:474–488.
22. **McNab, J. C., and M. C. Timbury.** 1976. Complementation of ts mutants by a herpes simplex virus ts-transformed cell line. Nature (London) **261**:233–235.
23. **Mes, A.-M., and J. A. Hassell.** 1982. Polyoma virus middle T-antigen is required for transformation. J. Virol. **42**:621–629.
24. **Muller, W. J., C. R. Mueller, A.-M. Mes, and J. A. Hassell.** 1983. Polyomavirus origin for DNA replication comprises multiple genetic elements. J. Virol. **47**:586–599.
25. **Muller, W. J., M. A. Naujokas, and J. A. Hassell.** Polyomavirus-plasmid recombinants capable of replicating have an enhanced transforming potential. Mol. Cell. Biol. **3**:1670–1674.
26. **O'Hare, K.** 1981. Replication of polyoma plasmid recombinants in mouse cells. J. Mol. Biol. **151**:203–210.
27. **Peden, K. W. C., J. M. Pipas, S. Pearson-White, and D. Nathans.** 1980. Isolation of mutants of an animal virus in bacteria. Science **209**:1392–1396.
28. **Pellegrini, S., L. Dailey, and C. Basilico.** 1984. Amplification and excision of integrated polyoma DNA sequences require a functional origin of replication. Cell **36**:943–949.
29. **Pomerantz, B. J., and J. A. Hassell.** 1984. Polyomavirus and simian virus 40 large T antigens bind to common DNA sequences. J. Virol. **49**:925–937.
30. **Pomerantz, B. J., C. R. Mueller, and J. A. Hassell.** 1983. Polyomavirus large T antigen binds independently to multiple unique regions on the viral genome. J. Virol. **47**:600–610.
31. **Pomerantz, B. J., M. Naujokas, and J. A. Hassell.** 1983. Homologous recombination between transfected DNAs. Mol. Cell. Biol. **3**:1680–1685.
32. **Shiroki, K., and H. Shimojo.** 1971. Transformation of green monkey kidney cells by SV40 genome: the establishment of transformed cell lines and replication of human adenoviruses and SV40 in transformed cells. Virology **45**:163–171.
33. **Soeda, E., J. R. Arrand, N. Smolar, J. E. Walsh, and B. E. Griffin.** 1980. Coding potential and regulatory signals of the polyoma virus genome. Nature (London) **283**:445–453.
34. **Sompayrac, L. M., and K. J. Danna.** 1981. Efficient infection of monkey cells with DNA of simian virus 40. Proc. Natl. Acad. Sci. **78**:7575–7578.
35. **Southern, E. M.** 1975. Detection of specific sequences among fragments separated by agarose gel electrophoresis. J. Mol. Biol **98**:503–515.
36. **Sutcliffe, J. G.** 1979. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. **43**:77–90.
37. **Treisman, R., A. Cowie, J. Favalaro, P. Jat, and R. Kamen.** 1981. The structure of the spliced mRNA encoding polyoma virus early region proteins. J. Mol. Appl. Genet. **1**:83–92.
38. **Treisman, R., U. Novak, J. Favalaro, and R. Kamen.** 1981. Transformation of rat cells by an altered polyoma virus genome expressing only the middle T-protein. Nature (London) **292**:595–600.
39. **Tyndall, C., G. LaMantia, C. M. Thacker, J. Favaloro, and R. Kamen.** 1981. A region of the polyoma virus genome between the replication origin and late protein coding sequences is required in *cis* for both early gene expression and viral DNA replication. Nucleic Acids Res. **9**:6231–6249.
40. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization using dextran sulfate. Proc. Natl. Acad. Sci. U.S.A. **76**:3683–3687.
41. **Wigler, M. A., A. Pellicer, S. Silverstein, and R. Axel.** 1978. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. Cell **14**:725–731.