JOURNAL OF VIROLOGY, Apr. 1987, p. 1226–1239
0022-538X/87/041226-14$02.00/0

Vol. 61, No. 4

Overproduction of Polyomavirus Middle T Antigen in Mammalian Cells through the Use of an Adenovirus Vector

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Received 13 August 1986/Accepted 18 December 1986

To overproduce biologically active polyomavirus middle T antigen, we used an adenovirus vector and human 293 cells as hosts. Two helper-independent recombinant adenoviruses were isolated that contain a hybrid transcription unit, in differing orientations, at a site in the adenovirus genome from which the E1a and most of the E1b transcription units have been deleted. The hybrid transcription unit consists of the adenovirus type 2 major late promoter and tripartite leader and a cDNA segment capable of encoding polyomavirus middle T antigen and accompanying 3′ RNA-processing signals. Both recombinant viruses were stable and replicated to high titers in human 293 cells. The polyomavirus sequences were expressed, predominantly at late times after infection of 293 cells, to yield mRNAs that encoded middle T antigen. One of the recombinant viruses also expressed a middle T antigen-related protein in 293 cells. The latter was translated from one of several novel mRNA species that resulted from aberrant splicing and incomplete RNA processing of precursor RNA transcripts. Comparison of the amount of middle T antigen produced in 3T6 cells infected with polyomavirus with that in 293 cells infected with either of the recombinant adenoviruses, under optimal conditions for each system, revealed at least a 10-fold greater yield of the protein on a per-cell basis in the latter system than in the former. The recombinant-virus-encoded middle T antigen was biologically active, as evidenced by its ability to associate with and serve as a substrate for human pp60$^{c\text{-}src}$. The functionality of the middle T antigen was further confirmed by demonstrating that both recombinant viruses efficiently transformed Rat-1 cells. These recombinant viruses will be useful to overproduce middle T antigen and to introduce the polyomavirus oncogene into a wide variety of mammalian cells.

The three structurally related polyomavirus (Py) early protein large, middle, and small tumor (T) antigens perform a number of functions required for virus replication and oncogenic transformation (64). During productive infection of permissive mouse cells in culture, large T antigen acts to repress early mRNA synthesis (16), to initiate viral DNA replication (26), and to stimulate late viral mRNA synthesis and cellular DNA replication (84). Although much less is known of the functions of middle and small T antigen during the lytic cycle, both of these proteins are required for efficient virus replication. Middle T antigen is required for virus assembly (28, 86), whereas small T antigen acts to stimulate viral DNA replication (48, 57, 79).

Transformation of cells in culture and induction of tumors in animals also require the action of the early proteins. Middle T antigen plays a critical role in both processes and represents the principal oncogene product of the virus (55, 85). It is absolutely required to transform both established (58, 85) and embryonic cells in culture (62) and to induce tumors in newborn rodents (2, 3, 12). In some experimental systems middle T antigen is sufficient to cause both of these events. These include transformation of embryonic chicken cells (40, 43) and induction of tumors in chickens (43), newborn hamsters (3), and mice (V. Bautch and J. A. Hassell, unpublished). Large T antigen and small T antigen play ancillary roles in oncogenesis. Large T antigen immortalizes rat embryo fibroblasts (63) and complements middle T antigen for full transformation of these cells (62), whereas small T antigen complements middle T antigen for tumor induction in newborn rats (2).

Because of its prominent role in oncogenesis, much attention has been focused on middle T antigen. Middle T antigen is a phosphoprotein (23, 65, 67, 73) which is located in the membrane fraction of infected and transformed cells facing the cytoplasm (36, 37). It is composed of 421 amino acids, yielding a predicted molecular weight of 48,506 (27, 75). Two species of middle T antigen with measured molecular weights of 56,000 (56K) and 58K have been identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) that differ in their abundance and extent of phosphorylation (66, 67). Phosphorylation of middle T antigen occurs principally at serine and threonine residues in vivo (66, 67) and to a much lower extent at tyrosine residues (71). By contrast, in immunoprecipitates in vitro, middle T antigen is phosphorylated essentially at tyrosine residues, mainly tyrosines 315 and 322 (23, 65, 73).

The tyrosine-specific kinase activity displayed in vitro by middle T antigen is not intrinsic (68, 69) but is due to its interaction with pp60$^{c\text{-}src}$ (17, 20, 21). Courtneidge and Smith (20) have shown that a small fraction of middle T antigen forms a stable complex with pp60$^{c\text{-}src}$, the product of the cellular oncogene *src*, which is also found in the plasma membrane (18, 19). One consequence of their interaction is an increase in the pp60$^{c\text{-}src}$-specific kinase activity in vitro, which results in its autophosphorylation at a novel tyrosine residue in the amino-terminal region of the protein (10, 90). The increased tyrosine kinase activity of the middle T antigen-pp60$^{c\text{-}src}$ complex apparently results from the failure of a cellular tyrosine kinase to phosphorylate a carboxy-terminal tyrosine residue (tyrosine 527) in pp60$^{c\text{-}src}$ (15). Middle T antigen binding to pp60$^{c\text{-}src}$ may block this reaction.

Despite a wealth of information about the nature of the middle T antigen-pp60$^{c\text{-}src}$ complex, it is not clear whether the increased tyrosine kinase activity of the complex measured in vitro is reflected in vivo. Apparently, the overall

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levels of phosphotyrosine in cellular proteins is not elevated in Py-transformed cells compared with their untransformed counterparts (70). We also do not know whether the increased tyrosine kinase activity of $pp60^{c\text{-}src}$ per se is important, or whether its specificity is also altered after interaction with middle T antigen (14). Moreover, much remains to be learned about the molecular mechanism by which middle T antigen and $pp60^{c\text{-}src}$ interact with each other and how their interaction brings about cellular transformation. Finally, middle T antigen associates with at least one other cellular protein (31), and the biological significance of this association is also unknown.

Elucidation of the mechanism of action of middle T antigen could be aided by the availability of large amounts of the protein. Middle T antigen is the product of a minimally expressed gene and represents only about 0.01% of the total cellular protein in Py-infected cells. To overcome this problem, we explored the use of adenovirus vectors and mammalian cell hosts, because this system has been successfully used to overproduce papovavirus tumor antigens (29, 50, 52, 76, 81–83, 87). We used an adenovirus type 5 (Ad5) vector to achieve enhanced expression of middle T antigen in human 293 cells. Two helper-independent recombinant viruses, named Ad5PyMTR1 and Ad5PyMTR2, were constructed by in vitro and in vivo recombination and characterized. These viruses contain a hybrid transcription unit composed of the Ad2 major late promoter and tripartite leader module joined to the coding sequences for Py middle T antigen and accompanying 3′ RNA-processing signals. The recombinant viruses contain the hybrid transcription unit in one or the other orientation at a site in the vector from which the E1a and most of the E1b transcription units have been removed. Human 293 cells infected with either recombinant virus yielded a 10-fold-higher level of middle T antigen than in 3T6 cells infected with Py under optimal conditions for each virus-cell system. Analysis of Py middle T-associated properties (kinase activity and transforming capacity after infection of Rat-1 cells) confirmed the functional nature of the middle T protein synthesized by the two recombinant viruses.

MATERIALS AND METHODS

Cells and viruses. All cell lines were propagated in plastic tissue culture dishes in Dulbecco modified Eagle medium (DMEM) supplemented with gentamicin (50 μg/ml), fungizone (2.5 μg/ml), and either 10% calf serum (human 293 and HeLa cells), 10% fetal bovine serum (FBS) (mouse 3T6 and Rat-1 cells), or 5% FBS (Rat-1 transformed cell lines). The cells were maintained at 37°C in a humidified CO_2 incubator.

Py stocks were prepared by infection of primary baby mouse kidney cells at a low multiplicity of infection (MOI) (0.01 to 0.1 PFU per cell), and virus was harvested 10 to 14 days postinfection. Recombinant viruses (Ad5PyMTR1 and Ad5PyMTR2) were propagated in 293 cells by infecting them at an MOI of 10 PFU per cell and harvesting the cells and medium 48 to 72 h afterward. Ad5*dl*309 was propagated similarly in HeLa cells (38). Virus was released from infected cells by three cycles of freezing and thawing and titrated by plaque assay.

Construction of the pPyMT recombinant plasmids. All cloning procedures were performed as described by Maniatis et al. (49). Recombinant plasmid DNAs were isolated from *Escherichia coli* DH1. Restriction enzymes, DNA-modifying enzymes, and synthetic DNA linkers were used according to the conditions specified by their manufacturers.

The plasmid pMPCV-2 (47) was the central vector for all the constructs (Fig. 1). pMPCV-2, a pML-2 derivative, contains the left-end 356 base pairs (bp), or 0 to 1 map unit (m.u.) of the Ad5 genome, a module composed of the Ad2 major late promoter, the entire first and second leaders, and two-thirds of the third leader (a cDNA fragment of Ad2 sequences from the *Sac*II site at 16.2 m.u. to the *Xho*I site at 26.5 m.u. lacking the naturally occurring splice sites and introns) (47, 91), and Ad5 sequences (from 7.9 to 15.5 m.u.) with two unique sites (*Xho*I and *Bgl*II) convenient for insertion of foreign DNA. A middle T-antigen cDNA fragment (85) devoid of the early promoter but containing the Py polyadenylation signals and site was assembled by using the plasmids pPyMTH(X)R and pPyH(S)B as illustrated in Fig. 1A. This fragment, which extends from an *Xho*I linker at nucleotide 154 (a former *Hph*I site [25] 19 bp upstream of the T-antigen initiation codon) to an *Hin*dII site at nucleotide 2964 (34 bp downstream from the polyadenylation site at nucleotide 2930) in Py DNA (numbering of Soeda et al. [75] was used), was modified by the addition of a *Bgl*II linker at the *Hin*dII site and cloned into pMPCV-2 immediately downstream from the Ad2 major late promoter and leader sequences, creating a hybrid transcription unit. Note that the middle T-antigen intron (nucleotides 746 through 809) is missing from the Py sequences. The resulting plasmid was designated pPyMT-1 (Fig. 1A).

Because we wished to construct recombinant viruses with alternate orientations of Py sequences, we used the plasmid pPyMT-1 to construct a second recombinant plasmid with the hybrid transcription unit in the opposite direction (Fig. 1B). The plasmid pPyMT-1 was digested with restriction endonuclease *Sac*II and blunt-ended with the Klenow fragment of *E. coli* DNA polymerase I (using the 3′-to-5′ exonuclease and polymerase activity of the enzyme), and *Bgl*II linkers were added to the ends. The fragment with a *Bgl*II site at each end was isolated and inserted into a derivative of pMPCV-2 at a newly generated *Bgl*II site. The latter plasmid was obtained, after excision of the sequences between the *Sac*II site (at nucleotide position 356 in Ad5 DNA) and the unique *Bgl*II site in pMPCV-2, by partial cleavage with *Sac*II, isolation of the appropriate fragment by agarose gel electrophoresis, treatment with the Klenow fragment of *E. coli* DNA polymerase I to blunt the ends, and ligation of *Bgl*II linkers to the ends. Screening by restriction endonuclease analysis allowed identification of the recombinant plasmid with the desired orientation; it was designated pPyMT-2.

Construction of Ad5PyMT recombinant viruses. The Ad-Py recombinant viruses were isolated by in vivo homologous recombination between linearized plasmid DNA and a large right-end fragment of Ad5*dl*309 DNA (6, 78). Four micrograms of pPyMT-1 or pPyMT-2 DNA was cleaved with *Cla*I, mixed with 4 μg of agarose gel-purified fragment A (3.8 to 100 m.u.), obtained by digesting Ad5*dl*309 DNA (38) with *Xba*I, and ethanol precipitated with 8 μg of calf thymus DNA and NaCl (0.1 M final concentration). Each DNA mixture was suspended and transfected onto two 60-mm dishes of 293 cells that had been plated the day before at a density of 6×10^5 cells per plate, using calcium phosphate as a facilitator of DNA uptake (30, 89). Eight hours later the DNA was removed, and the cells were washed with phosphate-buffered saline (PBS) and overlaid with either medium or agar for immediate plaque isolation.

At 8 to 12 days after transfection, plaques were isolated directly from the dishes overlaid with agar, and lysates were harvested from the plates that had not been overlaid with


pPyMT H(X)R
PyV mTcDNA fragment
Xho I
Eco RI
Eco RI digest, Ciap
Ligation
pPyH(S)B
Genomic fragment of PyV
Sal I
pBR322
Bam HI
Eco RI digest
pMPCV-2
MLP+L1
L2+3L3
Bgl II
Xho I digest
Bgl II digest
Hind III digest
Bgl II linkers, digest
Xho I-digest
Bgl II (Hind III)
pPyMT-1
Cla I digest
Sac II partial digest
Klenow
Elution of 1-cut molecules
Bgl II linkers, digest
Religation & screen for
Bgl II(Sac II/Bgl II)
Sac II digest
Ciap
Sac II total digest
Bgl II linkers, digest
Bgl II(Sac II)
mTcDNA
Ligation & screen for wanted orientation
pPyMT-2
Cla I/Eco RI
Sac II
Bgl II
Sal I/Xho I
PyMTcDNA
Ad5(dl309)/Xba I 'A' fragment
3.8 m.u.
Co-transfection in 293 cells and in vivo recombination
9.4 m.u.
15.5 m.u.
100 m.u.
0 m.u. 1 m.u.
Ad5-PyMTR1
Ad5-PyMTR2
A
B

agar. The latter were serially diluted and used to generate more plaques. Individual plaques were picked and frozen and thawed three times, and about 25 of them were screened. The desired recombinants were identified by extracting DNA from a 10% volume of the viral lysates (typically 200 μl) by the Hirt (34) procedure. The DNA was digested with proteinase K (at a final concentration of 1 mg/ml) and RNase (20 μg/ml) and purified by sequential phenol and chloroform-isoamyl alcohol (24:1) extraction and ethanol precipitation. The precipitates were suspended in 10 mM Tris hydrochloride, pH 7.9, and digested with *Hin*dIII to identify recombinant adenoviruses containing Py sequences. The DNA fragments were separated by electrophoresis through agarose gels and detected by ethidium bromide staining. The recombinant viruses, identified by their unique digestion pattern, were plaque purified twice more on 293 cells, and virus stocks were prepared and titrated.

For detailed analysis, viral DNA was prepared from 293 cells infected with individual recombinant viruses at an MOI of 20 PFU per cell. The DNA was isolated from CsCl gradient-purified virions by proteinase K digestion (60) and characterized by restriction endonuclease cleavage, Southern blotting (77), and hybridization with ^{32}P-labeled, nick-translated Py- or Ad-specific DNA probes (88). After being washed, the nitrocellulose sheets were dried and autoradiographed for 6 to 24 h at −70°C with Kodak XAR-5 film and DuPont Cronex Lightning-Plus intensifying screen.

RNA isolation. Total cytoplasmic RNA was isolated at either 8 h (early) or 15 h (late) postinfection from 293 cells infected with either recombinant virus or Ad5*dl*309 at an MOI of 20 PFU/cell. For comparison, 3T6 cells were infected with Py at an MOI of 100 PFU per cell, and RNA was isolated after 20 h (24, 49). Polyadenylated [poly(A)$^+$] RNA was selected by chromatography on oligo(dT)-cellulose columns (4) and stored as an ethanol precipitate at −20°C after evaluation of its concentration by absorbance at 260 nm.

Northern analysis. The variety and size of various mRNA species were analyzed by Northern blotting and hybridization with appropriate DNA probes. Generally, 1 to 5 μg of poly(A)$^+$ RNA was glyoxylated in a 16-μl reaction mixture containing 1 M deionized glyoxal (13), 50% (vol/vol) dimethyl sulfoxide, and 10 mM sodium phosphate buffer (pH 6.5) (80). The reaction mixture was incubated for 1 h at 50°C and cooled rapidly on ice, and 4 μl of sample buffer (50% [vol/vol] glycerol, 10 mM sodium phosphate, 0.4% bromophenol blue) was added. The denatured RNA was electrophoresed through a 1% agarose gel in 10 mM phosphate buffer at a constant pH of 6.5 to 7.0, transferred to nitrocellulose, and hybridized as described by Thomas (80). Restriction endonuclease-cleaved DNA was denatured with glyoxal in a similar manner and used as a molecular weight marker (54). Hybridizations were performed with ^{32}P-labeled DNA probes, prepared by nick-translation, at a specific activity of about 10^8 cpm/μg. The probes included the following DNAs: (i) Py fragment (nucleotides 154 to 1560); (ii) a *Bgl*II-*Sac*II Ad5 fragment (9.4 to 10.2 m.u.); (iii) Ad5 *Sac*II fragment (0 to 1 m.u.); (iv) Ad5 *Hin*dIII B fragment (72.8 to 89.1 m.u.); and (v) Ad5 *Hin*dIII C fragment (17.0 to 31.5 m.u.).

Radiolabeling and preparation of cellular extracts. Confluent monolayers of 293 cells at a density of about 6 × 10^6 cells per 100-mm plate were independently infected with either recombinant virus (Ad5PyMTR1 and Ad5PyMTR2) or with Ad5*dl*309 at an MOI of 20 PFU per cell. At early (8 h) or late (15, 18, and 21 h) times after infection, the cells were washed and labeled for 2 h with 125 μCi of [^{35}S]methionine (ca. 1,000 Ci/mmol) in 3 ml of methionine-free medium in which the cells had been incubated for 1 h prior to labeling. Concurrently, 3T6 cells at the same density were infected with Py at an MOI of 100 PFU per cell and processed similarly at 20 h postinfection. The cells were scraped off the dishes, washed twice with ice-cold PBS, and lysed by incubation for 30 min at 0°C in 500 μl of 100 mM Tris hydrochloride (pH 8.8)–100 mM NaCl–0.5% (vol/vol) Nonidet P-40 containing three protease inhibitors (200 μg of phenylmethylsulfonyl fluoride, 2 μg of aprotinin, and 10 μg of leupeptin per ml). Cellular debris was removed by centrifugation for 2 min in an Eppendorf centrifuge, and the supernatant was divided into portions, which were either used directly for immunoprecipitation or stored at −70°C.

Immunoprecipitation. Immunoprecipitation of Py T antigens synthesized in 293 or in 3T6 cells was performed as described previously (36) with modifications (35). A portion of the extract was adjusted with an appropriate volume of 20% SDS to a final concentration of 0.7%. This sample was boiled for 2 min and diluted 1:23 with cold Triton buffer (2% Triton X-100, 50 mM Tris hydrochloride [pH 8.0], 150 mM NaCl, and 0.1 mM EDTA containing the three protease inhibitors), thereby reducing the SDS concentration to 0.03%. This diluted extract was mixed with normal rat serum from brown Norwegian rats for 3 h at 0°C. A 20% suspension of Formalin-fixed *Staphylococcus aureus* Cowan I in Triton buffer, prepared as described by Kessler (41), was added, using 10 volumes of *S. aureus* cells per volume of serum. After incubation for 1 h on ice with occasional agitation, the samples were centrifuged in an Eppendorf centrifuge for 1 min at 4°C. The supernatant was removed and incubated with an excess of rat anti-Py tumor serum for 15 h at 0°C. The immunocomplexes were collected by centrifugation after incubation with 10 serum volumes of *S. aureus* as described above, washed twice with Triton buffer and once with 10 mM Tris hydrochloride (pH 8.0), and finally eluted from the bacteria by incubation for 30 min at room temperature with an appropriate volume (50 to 100 μl) of 7 M urea in 80 mM Tris hydrochloride (pH 8.0). The bacteria were removed by centrifugation, the supernatant was diluted 10 times with Triton buffer, and the concentrations of Triton X-100 and SDS were adjusted to 2% and 0.03%, respectively. The antigen-antibody complexes were allowed to reform overnight at 0°C and then adsorbed to Formalin-fixed *S. aureus*, pelleted, and washed as described previously. The final precipitate was suspended in sample buffer (0.0625 M Tris hydrochloride [pH 6.8], 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue), left for 15

FIG. 1. Construction and structure of the recombinant plasmids pPyMT-1 and pPyMT-2 and the recombinant adenoviruses Ad5PyMTR1 and Ad5PyMTR2. The two plasmids pPyMT-1 (A) and pPyMT-2 (B) were constructed by the experimental protocols described in Materials and Methods. Both contain a hybrid transcription unit comprising the Ad2 major late promoter (MLP) and most of the tripartite leader sequences (stippled box) juxtaposed to the sequences encoding the Py middle T antigen (open box), flanked by two Ad fragments (thick line), from 0 to 1 m.u. and from 9.4 to 15.5 m.u., within pML-2 DNA (thin line). Cotransfection of 293 cells with either of these recombinant plasmids that had been linearized with *Cla*I and the isolated Ad5*dl*309 *Xba*I A fragment allowed in vivo recombination between the homologous 9.4 to 15.5 m.u. DNA segment in each DNA to yield the recombinant viruses Ad5PyMTR1 and Ad5PyMTR2.

min at room temperature, boiled for 3 min, and centrifuged for 2 min at 20°C. The supernatant was loaded on a 10% SDS-polyacrylamide gel (44) and electrophoresed. Gels were fixed and stained with Coomassie brilliant blue (0.3%) in methanol-acetic acid-H_2O (5:1:4) for 30 min, destained for 8 h in the same solution, and impregnated with En^{3}Hance (New England Nuclear) as specified by the manufacturer. Gels were then dried, and the ^{35}S-labeled proteins were visualized by fluorography.

To analyze the structure of the T antigen-related protein, four rat monoclonal antibodies, one mouse monoclonal antibody, and one rabbit peptide monospecific antiserum were used. The four rat monoclonal antibodies are directed against specific determinants of middle T (αPyMT7 and αPyMT16) or large T antigen (αPyLT1 and αPyLT7) (22). The F4 mouse monoclonal antibody recognizes an amino-terminal epitope common to all three T antigens (E. Harlow, personal communication). The monospecific rabbit antiserum is directed against a synthetic peptide corresponding to 10 amino-terminal amino acids ([Tyr]-Asp-Lys-Glu-Arg-Leu-Leu-Glu-Leu-Leu-Lys) (amino acids 9 through 18) common to all three T antigens and was designated common T (cT) (B. Massie, B. Bugler, and J. A. Hassell, unpublished). Immunoprecipitation with these sera was carried out essentially as described by Dilworth and Griffin (22).

In vitro protein kinase assay. The technique described by Schaffhausen and Benjamin (66) was used to measure the protein kinase activity associated with middle T antigen. Human 293 cells were infected independently with either one of the two recombinant viruses or with Ad5*dl*309. For comparison, 3T6 cells were infected with Py as described above. The cells were harvested at 15 h postinfection with the recombinant adenoviruses or at 20 h postinfection with Py, washed twice in ice-cold PBS, and rinsed with 0.137 M NaCl–0.02 M Tris hydrochloride (pH 9.0)–0.001 M $MgCl_2$–0.001 M $CaCl_2$. The cells were lysed by incubation for 30 min at 4°C with 1 ml of the same buffer containing 10% glycerol and 1% Nonidet P-40. Extracts were cleared by centrifugation, and the supernatants were frozen at −70°C or used directly for the kinase reaction. Proteins were immunoprecipitated from 200 μl of extract, corresponding to an equal number of infected 293 or 3T6 cells, by incubation with 25 μl of rat antitumor serum and 200 μl of a 50% suspension of *S. aureus* protein A-Sepharose (Pharmacia) for 30 min at 4°C. The immunoprecipitates were collected, washed three times with cold PBS, twice with 0.5 M LiCl–0.1 M Tris hydrochloride (pH 6.8), and once with distilled water, and suspended in 40 μl of 0.02 M Tris hydrochloride (pH 7.5)–0.005 M $MgCl_2$ containing 25 μCi of [γ-^{32}P]ATP. The mixture was incubated for 15 min at room temperature. The precipitates were collected, washed as described above, suspended in 50 μl of sample buffer, boiled for 3 min, and electrophoresed through 10% SDS-polyacrylamide gels as described by Laemmli (44). Gels were stained, destained, dried, and exposed as described above, and the ^{32}P-labeled proteins were visualized by autoradiography.

Transformation assays. Rat-1 cells were plated on 100-mm plates so that the density was 2×10^6 cells after 24 h. Cells were infected independently with the two recombinant viruses, Ad5PyMTR1 and Ad5PyMTR2, as well as with Ad5ΔE1/*dl*309, which lacks early region E1a and most of E1b (29), at multiplicities of 1,000, 100, 10, 1, 0.1, and 0.01 PFU per cell. A set of three plates was infected at each MOI. Virus adsorption was carried out for 1 h at 37°C in 1 ml of DMEM, rocking the plates every 15 min. The virus was removed, the cells were washed twice with PBS, and 10 ml of DMEM containing 10% FBS was added. The medium was then changed every 3 days. Foci appeared by 10 days postinfection. After 3 to 4 weeks, the cells were fixed with 10 ml of 10% (vol/vol) buffered Formalin for 20 min and stained with Giemsa to visualize the foci.

To establish transformed cell lines, independent foci were isolated from separate plates and transferred to a 35-mm plate containing medium. When the plates were confluent, the cells were removed, diluted, and replated to allow isolation of individual colonies. A total of eight independent colonies, generated after infection with each recombinant virus, were established and characterized by immunoprecipitation as described.

RESULTS

Construction and genome structure of A5PyMT recombinant viruses. To achieve high-level expression of Py middle T antigen, a hybrid transcription unit was constructed containing a DNA segment from the Ad2 genome bearing the major late promoter and a cDNA copy of the Ad2 tripartite leader (91) joined to Py sequences capable of encoding only middle T antigen (85) (Fig. 1). The joint between the two viral sequences occurred at an *Xho*I site within the third adenoviral leader and 5′ untranslated Py sequences. The remaining Py sequences span the region between nucleotide 154 (joined to an *Xho*I linker) and nucleotide 2964, the location of a naturally occurring *Hin*dII site, which was converted to a *Bgl*II site. One component of the Py early polyadenylation signal, 5′-AATAAA-3′, occurs within this DNA segment, beginning at nucleotide 2915. The cDNA corresponding to the mRNA for middle T antigen used in these experiments lacks sequences between nucleotides 746 and 809, which correspond to the middle T antigen intron (85).

The hybrid transcription unit was placed in alternate orientations between 1.0 and 9.4 m.u. in Ad5*dl*309 as described in Materials and Methods to yield two recombinant viruses, Ad5PyMTR1 (Fig. 1A) and Ad5PyMTR2 (Fig. 1B).

The genomes of Ad5PyMTR1 and Ad5PyMTR2 were 0.5% larger than that of Ad5. Analysis of DNA obtained from virions by restriction endonuclease cleavage followed by agarose gel electrophoresis and Southern blotting and hybridization revealed that the two recombinant viruses were of the expected structure and had not undergone any deletions or rearrangements in either their Py or Ad sequences (data not shown). Moreover, the genomes of these recombinant viruses were stable, for no detectable changes in their DNA occurred at this level of resolution after three rounds of plaque purification and virus propagation. Finally, the inserted Py sequences did not detectably impair the rate of virus replication or the final virus yield of either recombinant virus by comparison with their parent, Ad5*dl*309, in human 293 cells (data not shown).

Py middle T antigen mRNAs transcribed from Ad5PyMT recombinant viruses. To determine the structure, abundance, and temporal order of expression of the Py middle T-antigen transcripts expressed from the recombinant virus genomes, we isolated cytoplasmic poly(A)$^+$ RNA at early (8 h) and late (15 h) times postinfection from Ad5PyMTR1- and Ad5PyMTR2-infected 293 cells and compared it with the cytoplasmic poly(A)$^+$ RNA isolated at 20 h postinfection from Py-infected 3T6 cells. The RNAs were first analyzed, after denaturation, by electrophoresis through agarose gels, transfer to nitrocellulose, and hybridization to a Py DNA probe from the early region (nucleotides 154 to 1560).

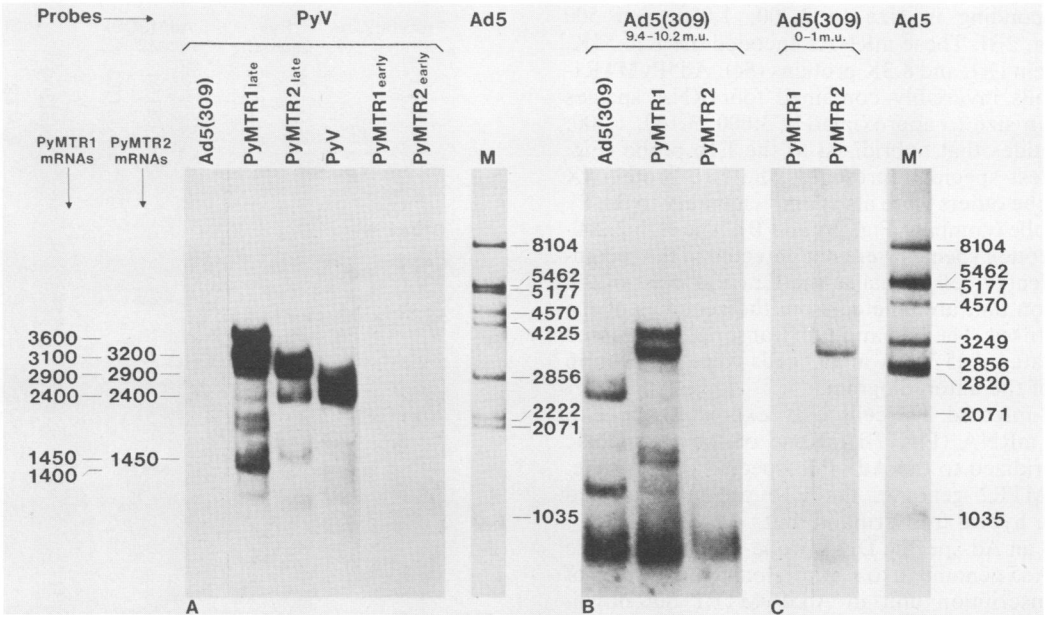

FIG. 2. Northern analysis of cytoplasmic poly(A)$^+$ RNA from 293 cells infected with the recombinant adenoviruses. One microgram of poly(A)$^+$ RNA extracted from 293 cells infected with Ad5*dl*309, Ad5PyMTR1, or Ad5PyMTR2 at 8 h (early) or 15 h (late) postinfection and a 10-μg sample of poly(A)$^+$ RNA from 3T6 cells infected with Py at 20 h postinfection were glyoxylated and fractionated on a 1% agarose gel. The RNA was transferred to nitrocellulose and hybridized with the following ^{32}P-labeled probes: Py DNA spanning the early region from nucleotide 154 to 1560 (A); Ad5*dl*309 DNA from m.u. 9.4 to 10.2 (B); or Ad5*dl*309 DNA from m.u. 0 to 1.0 (C). Ad5*dl*309 DNA (lane M) and Ad5PyMTR1 DNA (lane M′) were cleaved with *Hin*dIII, glyoxylated, and visualized by hybridization to ^{32}P-labeled Ad5 DNA to serve as molecular weight markers. Lane M served as a marker for panel A, whereas lane M′ served as a marker for panels B and C. The measured lengths of the Py-specific RNAs encoded by each recombinant adenovirus are indicated to the left of panel A (in nucleotides). Only those Py-specific RNAs observed in multiple repetitions of this analysis are marked.

The hybrid transcription unit incorporated in Ad5PyMTR1 and Ad5PyMTR2 should be expressed predominantly at late times after infection of 293 cells to yield a hybrid mRNA composed of 2,891 nucleotides (168 nucleotides representing the late Ad leaders, 10 nucleotides added at the Ad-Py junction, and 2,713 nucleotides from Py) if the Ad promoter signals and Py 3′ RNA-processing signals were used. Northern analysis of the poly(A)$^+$ RNA from 293 cells infected with the recombinant viruses revealed the presence of many Py-specific transcripts at late times postinfection (15 h) but none at early times postinfection (8 h) (Fig. 2A). Expectedly, no Py-specific transcripts were detected after infection of 293 cells by Ad5*dl*309 (Fig. 2A).

Six transcripts of approximately 3,600, 3,100, 2,900, 2,400, 1,450, and 1,400 nucleotides were invariably detected in 293 cells infected with Ad5PyMTR1, whereas four transcripts of 3,200, 2,900, 2,400, and 1,450 nucleotides were always detected in the same cells infected with Ad5PyMTR2 (Fig. 2A). The 3,600-, 3,100-, and 2,900-nucleotide RNAs from AdPyMTR1-infected cells were most abundant, whereas the 3,200- and 2,900-nucleotide RNAs from Ad5PyMTR2-infected cells represented the major species. The 293 cells infected with Ad5PyMTR1 also contained transcripts that were 2,200, 2,000, and 1,000 nucleotides long, but these were not always detected in different RNA preparations (data not shown). Their nature remains unknown.

Only one of the RNA species, 2,900 nucleotides long, corresponded in length to that predicted for the hybrid mRNAs (2,891 nucleotides). The 2,900-nucleotide RNA species accounted for about 15 to 20% of the total Py-specific RNA in 293 cells infected with Ad5PyMTR1, whereas it represented about 50 to 60% of the Py-specific RNA found in Ad5PyMTR2-infected 293 cells.

3T6 cells infected with Py contained three early mRNAs encoding large, middle, and small T antigen. Two RNA bands corresponding in length to approximately 2,700 and 2,400 nucleotides were observed (Fig. 2A). The largest RNA species was a mixture of the small and middle T-antigen mRNAs, whereas the smaller species represented the mRNA for large T antigen (32, 39). Comparison of the total amount of Py-specific RNA obtained from 293 cells infected with the recombinant viruses with that obtained from Py-infected 3T6 cells revealed a 10- to 15-fold-higher level in the recombinant-virus-infected cells (the low and high in three experiments). These comparisons were made when the steady-state level of T-antigen mRNA was highest in both virus-cell systems (52).

Several of the Py-specific transcripts synthesized in 293 cells infected with Ad5PyMTR1 and Ad5PyMTR2 were significantly larger than expected. Because there are no promoters other than the major late promoter proximal to the Py sequences in each recombinant virus, it seemed unlikely that these transcripts resulted from initiation events at cryptic promoters. We considered it more likely that the large transcripts resulted from inefficient use of the Py 3′ processing signals. If this were the case, then the Py-specific transcripts should contain Ad sequences at their 3′ ends derived from transcription of Ad5 DNA downstream from the Py sequences in each recombinant virus genome. To examine this, we hybridized blots of poly(A)$^+$ RNA from 293 cells infected with the recombinant viruses and Ad5*dl*309 with radiolabeled DNA probes that flank the inserted hybrid transcription unit in the recombinant viruses.

Hybridization of such a blot with an Ad5 DNA fragment from 9.4 to 10.2 m.u. (an E1b-specific probe) revealed that Ad5*dl*309-infected 293 cells contained three major RNA

species corresponding in size to 2,200, 1,000, and 500 nucleotides (Fig. 2B). These mRNAs encoded the E1b 55K, 21K, 14K (protein IX), and 8.3K proteins (84). Ad5PyMTR1-infected 293 cells invariably contained four RNA species corresponding in size to approximately 3,600, 3,100, 1,400, and 500 nucleotides that hybridized to the E1b probe (Fig. 2B). The smallest species represented the E1b protein IX mRNA, but all the others were also complementary to the Py early-region probe (compare Fig. 2A and B). The E1b 2,200- and 1,000-nucleotide species were not detected in the recombinant-virus-infected cells because the E1a and most of the E1b transcription unit are deleted from the genomes of the recombinant viruses. The E1a and E1b transcripts expressed from the integrated Ad5 DNA in 293 cells were not seen in this exposure of the autoradiogram.

Ad5PyMTR2-infected 293 cells only expressed the E1b 500-nucleotide mRNA (Fig. 2B). None of the Py-specific transcripts hybridized to the Ad5 E1b-specific DNA probe. In the Ad5PyMTR2 genome, the E1b region is located upstream of the hybrid transcription unit.

We also used an Ad-specific DNA probe from the extreme left end of the Ad5 genome (0 to 1 m.u.), located upstream of the hybrid transcription unit in Ad5PyMTR1 but downstream of it in Ad5PyMTR2. This region is not represented in poly(A)$^+$ cytoplasmic RNA from Ad5*dl*309-infected 293 cells (data not shown). Ad5PyMTR1-infected 293 cells also did not contain any cytoplasmic transcripts from this region of the Ad genome (Fig. 2C). By contrast, Ad5PyMTR2-infected 293 cells contained a single RNA species of 3,200 nucleotides that hybridized to the left-end Ad probe (Fig. 2C). This RNA corresponded in size to the largest species detected in Ad5PyMTR2-infected 293 cells with the Py-specific probe (Fig. 2A).

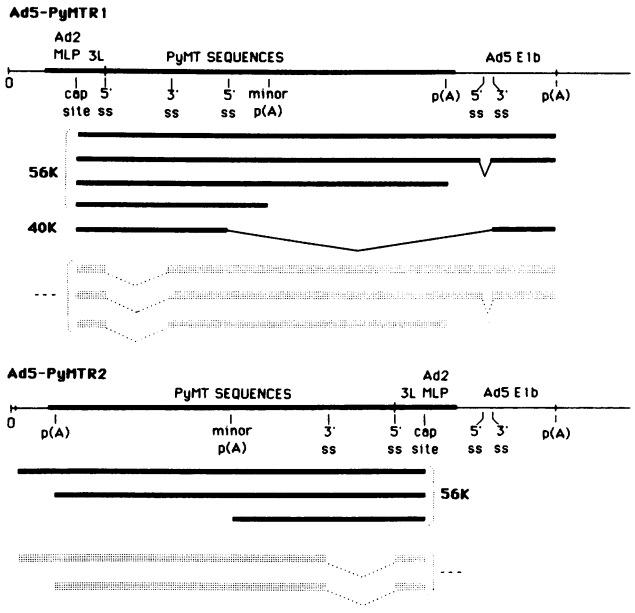

FIG. 3. Schematic representation of the cytoplasmic poly(A)$^+$ Py-specific RNAs synthesized in Ad5PyMTR1- and Ad5PyMTR2-infected 293 cells. A portion of the genomes of each recombinant virus is shown above the Py-specific RNAs which they encode. The thick line on the viral genomes represents the hybrid transcription unit. The locations of the cap site, 3′ and 5′ splice sites (ss), and polyadenylation [p(A)] sites are shown on the physical maps. The cytoplasmic poly(A)$^+$ RNAs encoded by the hybrid transcription unit in each recombinant virus are shown below the maps of the viral DNAs. The heavy lines represent RNAs which are thought to be translated to yield middle T antigen (56K) or a middle T antigen-related protein (40K). The RNAs identified by the stippled lines probably do not encode T antigen-related proteins, and it is not known whether they can be translated. The thin lines connecting the bodies of the spliced RNAs represent introns that are missing from the cytoplasmic poly(A)$^+$ RNAs. MLP, Major late promoter.

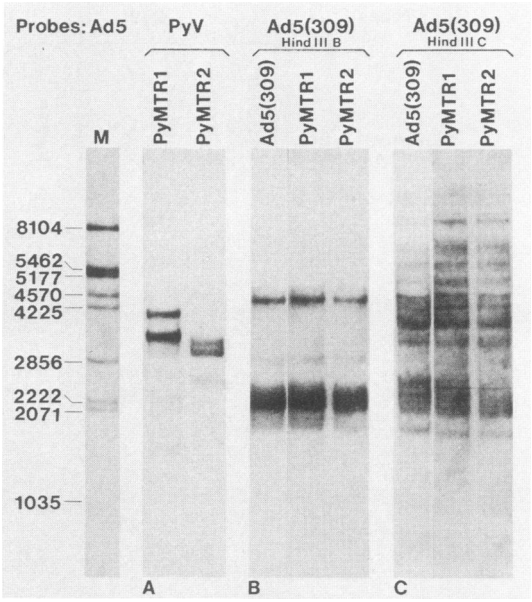

FIG. 4. Comparative Northern analysis of the abundance of the Py-specific and representative Ad late mRNAs synthesized in 293 cells infected with the recombinant Ads. One microgram of poly(A)$^+$ RNA prepared from 293 cells at 15 h postinfection with either Ad5*dl*309 or one of the recombinant viruses was analyzed as described in the legend to Fig. 2 by hybridization to the following probes: a Py DNA fragment from nucleotides 154 to 1560 (A); the *Hin*dIII B fragment of Ad5*dl*309 DNA (B); or the *Hin*dIII C fragment of Ad5*dl*309 DNA (C). Ad5*dl*309 DNA cleaved with *Hin*dIII and treated as described previously was used as a molecular size marker (lane M). Sizes are indicated (in nucleotides).

These results show that a significant proportion of the Py-specific transcripts in 293 cells infected with either recombinant virus failed to be processed at Py 3′-end processing signals. The two largest poly(A)$^+$ RNAs in Ad5PyMTR1-infected 293 cells (3,600 and 3,100 nucleotides long) may have resulted from initiation at the normal start site proximal to the major late promoter and 3′ processing near the E1b polyadenylation signal at nucleotide 4038 in Ad5 DNA. Similarly, the 3,200-nucleotide-long RNA in Ad5PyMTR2-infected 293 cells may have resulted from initiation at the normal late start site and 3′ processing downstream of potential polyadenylation signals on the L strand of Ad5 DNA. Nonetheless, the structure of several of the Py-specific transcripts could not be accounted for solely by inefficient 3′-end formation. To determine the structure of the individual Py-specific RNAs transcribed from the Ad5PyMTR1 and Ad5PyMTR2 genomes, we used S1 endonuclease (5) and a variety of paired, uniquely end-labeled DNA probes prepared from the recombinant plasmids pPyMT-1 and pPyMT-2 (data not shown). By taking into account the results of the Northern and S1 analyses we were able to deduce the structure of nearly all the recombinant-virus-encoded, cytoplasmic poly(A)$^+$ RNAs that were detected by Northern analysis (Fig. 3). All the RNAs appeared to have a common 5′ terminus, but differed at their 3′ termini

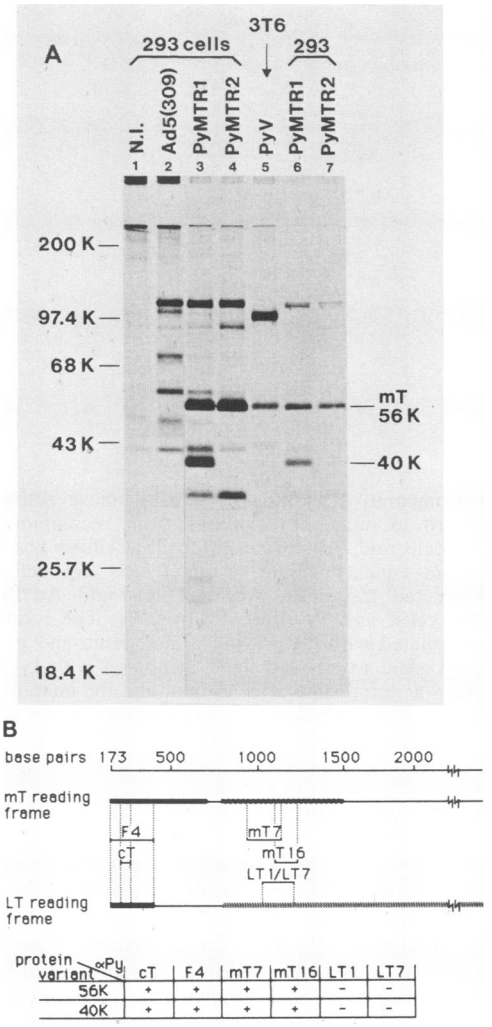

FIG. 5. Relative quantitation of Py middle T (mT) antigen synthesized in Ad5PyMTR1- and Ad5PyMTR2-infected 293 cells and Py-infected 3T6 cells, and structure of the 40K protein. (A) Cellular extracts were prepared from an equal number of uninfected cells (N.I., lane 1), Ad5*dl*309-infected 293 cells (lane 2), Ad5PyMTR1-infected 293 cells (lane 3), Ad5PyMTR2-infected 293 cells (lane 4), and Py-infected 3T6 cells (lane 5) that had been metabolically labeled with [^{35}S]methionine for 2 h at 15 h postinfection with the various Ads or at 20 h postinfection with Py. The extracts were immunoprecipitated and further processed as described in Materials and Methods. Lanes 6 and 7 contain one-tenth as much sample as was applied to lanes 3 and 4, respectively. (B) Summary of the results of immunoprecipitating the recombinant-virus-encoded T antigens with a variety of antibodies directed against them. The topmost line represents Py DNA beginning at the sequences encoding the initiation codon for the Py T antigens (nucleotide 173) and extending beyond the termination codon (nucleotide 1497) for middle T (mT) antigen. The next line down depicts the mRNA for middle T antigen. The black and braided boxes represent coding exons in different reading frames, and the thin line connecting them represents the middle T antigen intron. A similar drawing representing the large T (LT) antigen mRNA is shown below. The amino-terminal exon of large T antigen (black box) is in the same reading frame as that of middle T antigen, but its carboxy-terminal exon (stippled box) is not. Each antibody used in the analysis recognizes an epitope encoded by sequences within the areas shown. The results of the analysis are shown in the bottom table, indicating whether the protein was immunoprecipitated or not. Abbreviations: F4, mouse monoclonal antibody; cT, rabbit polyclonal antibody to a peptide which is common to all three T antigens; mT7 and mT16, two rat monoclonal antibodies that recognize epitopes only in middle T antigen; LT1 and LT7, two rat monoclonal antibodies that recognize epitopes unique to large T antigen.

and in internal sequences. The various 3′ ends resulted from the inefficient use of Py 3′-end formation and polyadenylation signals, whereas several cryptic splice sites within Py sequences were used to yield spliced RNAs.

To determine whether the hybrid mRNAs expressed from the recombinant viruses were as abundant as those corresponding to the major late Ad genes, we compared the steady-state levels of the Py-specific RNAs with the L4 and L5 families of RNA. This was accomplished by hybridizing blots of poly(A)$^+$ RNA prepared from cells infected with either Ad5*dl*309, Ad5PyMTR1, or Ad5PyMTR2 with a Py-specific DNA probe and independently with an Ad probe (the Ad5 *Hin*dIII B fragment) homologous to the late mRNAs for the 100K, 33K, pVIII (L4), and fiber (L5) proteins (Fig. 4A and B). They reveal that the major Py-specific RNAs from cells infected with either Ad5PyMTR1 or Ad5PyMTR2 (Fig. 4A) were as abundant as any of the individual late mRNAs (i.e., the largest Ad mRNA for the 100K protein in Fig. 4B) synthesized in the same cells or from 293 cells infected with Ad5*dl*309 (Fig. 4B). To ensure that equal amounts of poly(A)$^+$ RNA were compared in the experiments shown in Fig. 4A and B, the blots were also hybridized to a ^{32}P-labeled probe homologous to the Ad tripartite leader (the Ad5 *Hin*dIII C fragment). The results showed that essentially equal amounts of Ad-encoded poly(A)$^+$ RNA were compared (Fig. 4C).

Middle T antigen synthesis in Ad5PyMTR1- and Ad5PyMTR2-infected 293 cells. To determine whether middle T antigen was expressed in 293 cells infected with the recombinant viruses, we metabolically labeled cultures with [^{35}S]methionine for 2 h at various times postinfection and analyzed the products by SDS-PAGE after immunoprecipitation with rat polyclonal antiserum. We also analyzed the products expressed from Ad5*dl*309-infected 293 cells and uninfected 293 cells with the same antiserum as negative controls (Fig. 5A).

Expectedly, no proteins were immunoprecipitated from uninfected 293 cells, but several proteins, corresponding in size to Ad5-encoded late proteins, were immunoprecipitated, probably nonspecifically, from 293 cells infected with Ad5*dl*309 at 15 h postinfection. Two abundant T antigen species were expressed in 293 cells infected with Ad5PyMTR1, but not in cells infected with Ad5*dl*309. These proteins corresponded in size to 56K and 40K. The largest of them was the same size as authentic middle T antigen. Like the Py-specific RNAs encoded by Ad5PyMTR1, the T antigens (56K and 40K) were expressed at late (15, 18, and 21 h) but not at early (8 h) times after infection (data not shown). Ad5PyMTR2-infected cells synthesized only middle T antigen. The time of appearance and abundance of the 56K middle T antigen were the same in 293 cells infected with Ad5PyMTR2 or Ad5PyMTR1. The steady-state levels of middle T antigen were highest by 15 to 18 h postinfection and did not increase significantly after these times and the time of cell lysis, which occurred between 24 and 30 h postinfection.

Using optimal conditions for each virus-cell system, we compared the yield of middle T antigen on a per-cell basis from AdPyMTR1- and AdPyMTR2-infected 293 cells and from Py-infected 3T6 cells. The recombinant-virus-infected 293 cells clearly synthesized more middle T antigen than the Py-infected 3T6 cells (Fig. 5A, compare lanes 3 and 4 with

lane 5). The lysates from the infected human cells had to be diluted 10-fold to obtain the same amount of middle T antigen as yielded by the Py-infected 3T6 cells (Fig. 5A, compare lanes 6 and 7 with lane 5). In separate repetitions of this experiment the level of overproduction varied between 8- and 12-fold (data not shown).

Human 293 cells infected with Ad5PyMTR1 synthesized a novel 40K T antigen. To deduce the structure of this 40K T antigen we used five monoclonal antibodies and a monospecific serum, which recognize mapped epitopes on the Py T antigens, to immunoprecipitate the T antigens from 293 cells infected with Ad5PyMTR1 (Fig. 5B).

The monospecific rabbit serum (cT) recognizes an epitope between amino acids 9 and 18 that is common to all three T antigens (B. Bugler, B. Massie, and J. A. Hassell, unpublished). Similarly, the F4 mouse monoclonal antibody recognizes a determinant, within the 79 first amino acids, that is shared among the T antigens (E. Harlow, personal communication). Both of these antibodies immunoprecipitated the 40K T antigen. The monoclonal antibodies MT7 and MT16, which recognize antigenic determinants that occur only in middle T antigen, also immunoprecipitated the 40K T antigen. Because the epitope recognized by MT16 could be as far as 300 amino acids from the amino terminus of middle T antigen (22), these results indicate that the 40K species shares most of its sequences with middle T antigen, which is composed of 421 amino acids. The monoclonal antibodies LT1 and LT7, specific for large T antigen, did not immunoprecipitate the 40K T antigen. Together, these results suggest that the 40K T antigen is a truncated version of middle T antigen.

By taking into account the structure of the 56K middle T antigen and the 40K protein and that of the Py-specific RNAs, it is possible to assign mRNAs for each protein (Fig. 3). Four of the Py-specific RNAs expressed by Ad5PyMTR1-infected cells and three of those expressed by Ad5PyMTR2-infected cells could encode middle T antigen. Only one of the Py-specific RNAs expressed by Ad5PyMTR1-infected 293 cells, which hybridized to both Py and Ad5 E1b DNA, appeared to have a structure capable of encoding the 40K protein. This 1,400-nucleotide-long spliced RNA, like the 40K protein, was unique to Ad5PyMTR1-infected 293 cells. It is unlikely that the three structurally related Py-specific RNAs encoded by Ad5PyMTR1 possessing the novel splice located at the 5′ end of the Py middle T sequences and the two encoded by Ad5PyMTR2 could be translated to yield the 40K protein. All of these RNAs apparently lacked sequences capable of encoding the amino-terminal domain of either the 40K or 56K protein. We do not know whether any of these RNAs are translated in infected cells.

Because the 1,400-nucleotide spliced RNA was much less abundant than the middle T antigen mRNAs (Fig. 2A and B), whereas the two proteins they encode were equally abundant, we suggest that this RNA must be translated more efficiently than those that encode middle T antigen or that the 40K protein must be more stable than the 56K middle T antigen or both.

Biological activity of the recombinant-virus-encoded middle T antigens. Middle T antigen can serve as a substrate for pp60$^{c\text{-}src}$ in vitro in immunoprecipitates (20). Middle T antigen and pp60$^{c\text{-}src}$ form a stable complex, which can be immunoprecipitated with antiserum directed against either protein (20, 21). The tyrosine kinase-specific activity of the complex is enhanced by a factor of 10- to 20-fold compared with uncomplexed pp60$^{c\text{-}src}$ (10, 14, 15, 17). Py middle T antigen is known to form a complex with and activate the pp60$^{c\text{-}src}$ tyrosine kinase of mice (20), rats (21), hamsters (10), and chickens (40, 43). To determine whether the recombinant-virus-encoded middle T antigens could associate with human pp60$^{c\text{-}src}$ and act as a substrate for phosphorylation, we assayed immunoprecipitates obtained from infected 293 cells by incubation with rat anti-T polyclonal antibodies for their ability to phosphorylate middle T antigen (Fig. 6).

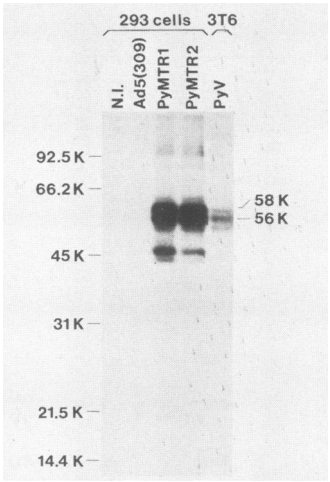

FIG. 6. Comparative analysis of protein kinase activity measured in vitro in immunoprecipitates from recombinant-virus-infected 293 cells and Py-infected 3T6 cells. Cellular lysates were prepared from equal numbers of uninfected 293 cells (N.I.), Ad5*dl*309-infected 293 cells, Ad5PyMTR1- and Ad5PyMTR2-infected 293 cells, and Py-infected 3T6 cells. The lysates were immunoprecipitated with rat polyclonal antiserum and protein A-Sepharose, washed extensively, and incubated with [γ-^{32}P]ATP. The reaction was terminated after 15 min, and the immunoprecipitates were washed again before they were electrophoresed through a 10% SDS-polyacrylamide gel and the labeled proteins were visualized by autoradiography. The positions of markers of known molecular weight are shown to the left of the autoradiograph, and those of the phosphorylated 58K and 56K forms of middle T antigen are shown to the right.

Extracts from uninfected and Ad5*dl*309-infected 293 cells did not yield phosphorylated forms of middle T antigen after incubation of immunoprecipitates with [γ-^{32}P]ATP. By contrast, immunoprecipitates from 293 cells infected with either Ad5PyMTR1 or Ad5PyMTR2 yielded two prominent, equally abundant species (56K and 58K) of phosphorylated middle T antigen. Several other smaller proteins, particularly a 47K species, were also phosphorylated. These smaller proteins may be degradation products of middle T antigen (66), or they may be cellular proteins that were associated with the immunocomplexes. A band corresponding to a 60K protein, more clearly visible in shorter exposures of the autoradiograph, was also phosphorylated in these reactions. It may correspond to human pp60$^{c\text{-}src}$, which is capable of autophosphorylation (59). It is noteworthy that the abundant 40K form of middle T antigen found in Ad5PyMTR1-infected 293 cells did not become phosphorylated in these immunoprecipitates, probably because this truncated protein lacks carboxy-terminal sequences which contain the principal sites of tyrosine phosphorylation (tyrosines 315 and 322) and the hydrophobic, membrane-anchoring domain.

The two forms of phosphorylated middle T antigen, 56K and 58K, were also found in Py-infected 3T6 cells after

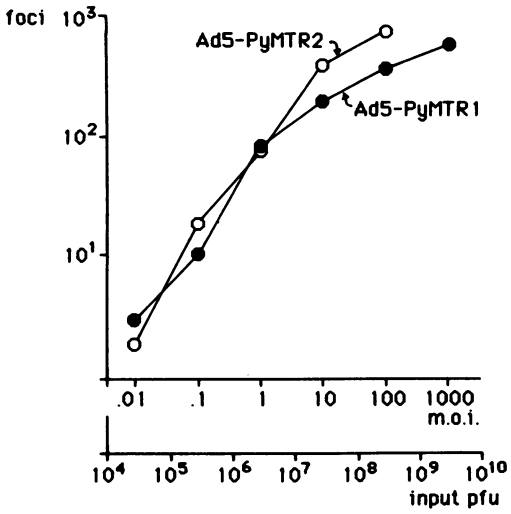

FIG. 7. Transformation of Rat-1 cells by Ad5PyMTR1 and Ad5PyMTR2. Rat-1 cells (2×10^6) were infected at differing multiplicities, and the cells were washed extensively and incubated with complete medium, which was replenished every 3 days. Three to 4 weeks after infection the cells were fixed with Formalin and stained with Giemsa, and the number of foci per plate was counted. Each point on the curves represents the average number of foci on three plates.

reaction of immunoprecipitates with [γ-^{32}P]ATP. In this experiment, the 56K form was phosphorylated to a greater extent than the 58K form, but this was not generally the case (data not shown). Usually, both forms are phosphorylated to the same extent (67). The abundance of the phosphorylated 56K and 58K species was at least 10 times higher in immunoprecipitates from recombinant-virus-infected 293 cells than in immunoprecipitates prepared from an equal number of Py-infected 3T6 cells. This result suggests that the amount of pp60$^{c\text{-}src}$ in 293 cells available for interaction with middle T antigen was not limiting.

There is a strong correlation between the capacity of mutant forms of middle T antigen to associate with and activate pp60$^{c\text{-}src}$ and their capacity to transform rat cells (9, 10, 14, 20). However, several Py mutants encode altered middle T antigens, which activate the pp60$^{c\text{-}src}$ kinase but do not transform cells (14, 56, 58). Because there are no known mutants with mutations affecting middle T antigen that transform cells but fail to activate pp60$^{c\text{-}src}$, it has been argued that association of the two proteins is necessary but not sufficient to effect transformation (14).

To ensure that the recombinant-virus-encoded middle T antigens were biologically active for transformation, we measured the capacity of these viruses to transform Rat-1 cells (Fig. 7). Ad5ΔE1/*dl*309, which lacks the E1a and most of the E1b transcription units corresponding to the same Ad sequences that are missing from the recombinant viruses, did not transform Rat-1 cells even at the highest MOI (1,000 PFU per cell). By contrast, both recombinant viruses transformed Rat-1 cells, as measured by the focus assay. The number of foci per plate increased linearly with increasing MOI over a wide range. There was no deleterious effect associated with infecting these rat cells at high MOIs.

Many independent Ad5PyMTR1- or Ad5PyMTR2-transformed cell lines, obtained after cloning the transformed Rat-1 cells from each focus, synthesized a protein that comigrated with Py middle T antigen (Fig. 8). A doublet was observed in each transformed cell line at the position of middle T antigen. These bands were likely the 56K and 58K species of middle T antigen.

Interestingly, the Ad5PyMTR1-transformed rat cell lines did not express the 40K middle T antigen. This protein could not be detected even after prolonged exposure of the fluorograph (data not shown). There was a 42K protein in these cells; however, it was also found in the untransformed parental Rat-1 cell line. It is a cellular protein which reacted with this particular preparation of antiserum. These results establish that the middle T antigen expressed by both recombinant viruses was biologically active as a substrate for phosphorylation by pp60$^{c\text{-}src}$ and as a transforming agent.

DISCUSSION

We have constructed and characterized two recombinant Ads, Ad5PyMTR1 and Ad5PyMTR2, that express Py middle T antigen. The recombinant viruses lack most of the E1 region and contain in its place a hybrid transcription unit composed of the Ad2 major late promoter and most of the tripartite leader fused to a Py cDNA capable of encoding middle T antigen and accompanying 3′ processing signals from the Py early region. The two recombinant viruses only differ by the orientation of the hybrid transcription unit within the Ad genome.

The genomes of both recombinant viruses were stable. No changes occurred after repeated plaque purification and passage of the virus stocks. Moreover, each virus replicated to levels indistinguishable from wild-type Ad2 or Ad5 in human 293 cells.

The hybrid transcription unit in each virus was expressed at late but not at early times after infection of 293 cells. However, both recombinant viruses were capable of efficiently transforming Rat-1 cells, suggesting that the Py

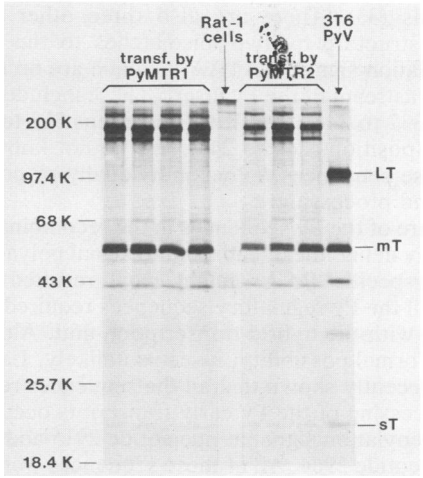

FIG. 8. Synthesis of middle T antigen by Rat-1 cells transformed by either Ad5PyMTR1 or Ad5PyMTR2. Seven independently transformed cell lines obtained by infection with either Ad5PyMTR1 or Ad5PyMTR2 were metabolically labeled with [^{35}S]methionine for 4 h, and cell lysates were prepared. The lysates were immunoprecipitated with rat polyclonal antiserum against Py T antigens, and the immunoprecipitated proteins were separated by electrophoresis through a 10% SDS-polyacrylamide gel. Lysates prepared from Py-infected 3T6 cells and analyzed similary served as a positive control (last lane), whereas lysates from untransformed Rat-1 cells served as a negative control. The positions of molecular weight markers are shown to the left, and those of large (LT), middle (mT), and small (sT) T antigen are shown to the right.

middle T antigen could be expressed in the early phase of infection prior to the initiation of viral DNA replication. (The recombinant viruses lack early-region E1a and most of E1b and consequently do not replicate their DNA after infection of any cell other than those that provide these functions in *trans*). There are at least two explanations for this apparent contradiction. First, it is known that the Ad2 major late promoter is active, albeit weakly, early after infection of human cells (1, 46, 72). Second, the products of the E1a transcription unit, provided by the 293 cells, are thought to block the activity of the E1a enhancer (11, 74), which is retained in both recombinant viruses. We suggest that early after infection of 293 cells the hybrid transcription unit is not expressed efficiently because the E1a products repress its transcription. However, in Rat-1 cells, expression of this transcription unit is possible because the E1a enhancer and major late promoter are active and no E1a products are made.

Surprisingly, a wide variety of cytoplasmic poly(A)$^+$ RNAs were detected in 293 cells infected with each recombinant virus. These RNAs were all initiated from the major late start site downstream from the Ad2 late promoter. Their heterogeneity resulted from the failure of the Py early polyadenylation and 3′-end formation signals to function efficiently and from aberrant splicing events. More than half of the Ad5PyMTR1-encoded RNAs contained 3′ ends that were defined by 3′-processing signals from the Ad5 E1b transcription unit. Similarly, the 3′ termini of about one-third of the RNAs encoded by Ad5PyMTR2 appeared to be defined by sequences at the extreme left end of the Ad5 genome, which are not normally used in the synthesis of Ad transcripts, to signal 3′-end formation and polyadenylation. The sequence AATAAG, related in sequence to AATAAA, is found between nucleotides 18 and 23 on the adenoviral L strand, and this sequence is known to function, albeit poorly, to signal 3′-end formation and polyadenylation in human cells (33). There are also three other sequences related in structure by two mismatches to the consensus polyadenylation signal, AATAAA, which are on the viral L strand downstream of the Py insert. These include AATATT (positions 337 to 344), AATTAT (positions 313 to 318), and AATTTA (positions 241 to 246). We do not know whether any of these sequences, either individually or collectively, signal 3′-end processing.

The failure of the Py sequences in the recombinant viruses to efficiently define the 3′ ends and to signal polyadenylation of the Py-specific RNAs might have resulted from not including all the Py regulatory sequences required for 3′-end processing with the hybrid transcription unit. Although this remains a formal possibility, it seems unlikely. Lanoix et al. (45) have recently shown that all the sequences required for 3′-end processing of the Py early transcripts occur between the polyadenylation signal at nucleotide 2915 and an *Hin*dII site at nucleotide 2964. All of these sequences were included with the hybrid transcription unit present in both recombinant viruses. Another possibility is that early Py 3′ processing may be intrinsically weak in all mammalian cells or particularly weak in human 293 cells. Py normally replicates in murine cells, and its regulatory signals may have evolved to function more readily in these cells than in primate cells.

Whatever the explanation, one consequence of transcription beyond the Py 3′-processing signals was the formation of precursor transcripts with increased numbers of splice sites. For example, an mRNA unique to Ad5PyMTR1-infected cells was formed from a precursor whose 3′ end mapped in E1b by joining a cryptic 5′ splice site in Py sequences (in the vicinity of nucleotide 1150) to the 3′ splice site in E1b to yield an mRNA that encoded the 40K protein. Transcripts such as these deplete the pool of precursor RNAs that could be used to form middle T antigen mRNA. Similarly, we suspect that some of the RNAs derived by transcription of the Py sequences in Ad5PyMTR2 are not polyadenylated because their 3′ termini lack the appropriate processing signals. Efficient processing of these transcripts might further increase the amount of middle T-antigen mRNA expressed in Ad5PyMTR2-infected cells.

In addition, many of the cytoplasmic poly(A)$^+$ RNAs expressed from the hybrid transcription unit in each recombinant virus were formed by splicing, using sites in Py that have heretofore not been known to function as splicing signals. These splice sites were not mapped to the nucleotide, but we know that a 5′ splice site at or near nucleotide 154 was coupled to a 3′ splice site near nucleotide 670 in precursor RNAs expressed from both recombinant virus genomes. This splice occurred in transcripts whether their 3′ ends were defined by Py or Ad regulatory signals. The large T-antigen 5′ splice site at nucleotide 409 present in the precursor transcripts expressed from both recombinant genomes was not used. Therefore, only certain pairs of splice sites were used to form the various spliced transcripts. These aberrantly spliced transcripts also deplete the RNA precursor pool and consequently the amount of middle T antigen mRNA. It is not clear what accounts for the synthesis of these aberrantly spliced transcripts. Abnormal splices analogous to these have been observed in simian virus 40 (SV40) transcripts expressed from Ad5-SV40 hybrid viruses (42, 81–83) and from Py transcripts expressed from SV40-Py recombinant viruses (92). Moreover, the frequency with which normal papovavirus splice sites are used can also differ in human or monkey cells infected with Ad5-SV40 hybrids (81) and Ad5-Py hybrids (50). It is possible that Ad infection perturbs the cellular splicing machinery by reducing or altering its specificity (1, 72).

Assay of the levels of the major Py-specific RNAs in human cells infected with either recombinant virus revealed that these were as abundant as any individual late Ad mRNA. For example, the most plentiful hybrid mRNA was as abundant as the mRNA for the Ad5 100K protein. If all the factors governing the synthesis and stability of the Py-specific RNAs and the late Ad mRNAs were the same, then we would have expected the hybrid mRNAs to be as abundant as the sum of all the late Ad mRNAs. This was clearly not the case. Observations similar to these have been made for the mRNA for dihydrofolate reductase expressed from an Ad recombinant of similar structure (8). Recent results suggest that sequences in Ad located between those encoding the second and third members of the late tripartite leader affect the rate of transcription from the major late promoter (51). These transcriptional signals are absent from the ectopic late promoter in the Ad5PyMT recombinant viruses. If these sequences function as an enhancer of late transcription, then it may be possible to increase transcription from ectopic late promoters by including this putative enhancer or other ones in their vicinity.

Like the Py-specific RNAs, middle T antigen was synthesized after the onset of Ad DNA replication in 293 cells infected with each recombinant virus. Ad5PyMTR1 also encoded an abundant 40K protein, and we were able to show that this 40K protein was structurally related to middle T antigen. It is probably a fusion protein composed of the amino-terminal 300 amino acids of middle T antigen and an

unknown number of carboxy-terminal amino acids encoded by Ad5 E1b sequences.

The 56K middle T antigen encoded by each recombinant virus was biologically active, as evidenced by its capacity to associate with and serve as a substrate for pp60$^{c\text{-}src}$. On the other hand, the 40K middle T antigen-related protein did not serve as a substrate for pp60$^{c\text{-}src}$, presumably because the protein lacks the carboxy-terminal hydrophobic domain of middle T antigen and cannot be inserted into the plasma membrane where the interaction between middle T antigen and pp60$^{c\text{-}src}$ is thought to take place. However, it is also possible that the 40K protein cannot associate with the pp60$^{c\text{-}src}$ because it lacks a binding site for this protein. These results also show that the 40K protein cannot serve as an exogenous substrate in immunoprecipitates for the middle T antigen-pp60$^{c\text{-}src}$ complex.

Both recombinant Ads proved capable of transforming Rat-1 cells. In fact, they transformed Rat-1 cells at frequencies equivalent to that reported for Py, although under slightly different experimental conditions (61). Moreover, these recombinants transformed cells at frequencies equivalent to that of Ad5-SV40 recombinants of similar structure (87). Examination of the T antigens expressed in independent transformed cell lines obtained after infection with one or the other recombinant virus revealed that only the 56K middle T antigen was present. The 40K protein was not produced by any of the Ad5PyMTR1-transformed cell lines, probably because the spliced mRNA that yields the 40K protein is not synthesized in Rat-1 cells.

Comparison of the amount of middle T antigen obtained from human cells infected with the recombinant viruses and mouse 3T6 cells infected with Py under optimal conditions for each virus-cell system revealed that 10 times as much functional middle T antigen could be isolated from infected human cells. Both recombinant viruses overexpressed middle T antigen to essentially the same extent in these cells. The greater yield of biologically active middle T antigen offered by this system and the ability to grow 293 cells in suspension culture make it an attractive source of the protein.

Previous attempts to overproduce middle T antigen in *E. coli* have been relatively unsuccessful; this was due to the inherent toxicity of the protein and its instability in these bacteria (68). SV40 and Ad5-SV40 hybrid viruses have also been used in attempts to provide a richer source of middle T antigen. A late SV40 replacement vector was used to overproduce middle T antigen in CV-1 monkey cells (92), and an Ad5-SV40 vector was used to simultaneously overproduce all three Py T antigens in human HeLa cells (50). In neither case were the levels of overproduction of middle T antigen as great as those reported here. Moreover, these defective recombinant viruses or their helpers encode the SV40 T antigens and, in the case of the Ad5-SV40 hybrid vector, the Ad oncogene products as well. This makes them unsuitable as gene transfer vectors in studies of cellular transformation and oncogenesis mediated by the Py T antigens.

Although the two recombinant Ads described here are useful for overproducing middle T antigen and can function as gene transfer vectors, we believe it will be possible to improve them. Two problems which we suspect reduce the yield of middle T antigen are the failure of 293 cells to heed Py 3′-processing signals and the occurrence of aberrant splicing events. In some cases the former process promotes the latter by allowing the synthesis of large precursor RNAs containing many potential splice sites. The use of strong 3′-processing signals from other viruses or genes may partially alleviate this problem. Preliminary results suggest that the inclusion of SV40 3′-processing signals in the recombinant virus genomes increases middle T antigen production three- to fivefold (unpublished).

It is not obvious how aberrant splicing events can be suppressed when these occur within the inserted sequences in the Ad vector. If their occurrence reflects a requirement to splice RNA for efficient nuclear processing and transport to the cytoplasm, then it may be possible to provide a noncoding exon to the hybrid transcription unit to meet this condition (7, 8).

Finally, it should be possible to improve the ability of the recombinant viruses to act as gene transfer vectors. The use of Ads for this purpose has remained largely unexplored, primarily because retrovirus vectors now offer a better means of achieving efficient integration of foreign genes into cellular chromosomes. Ads do not have this ability, but it may be possible to endow them with this attribute. Human Ads possess many of the other features that make retroviruses appealing as vectors, and unlike retrovirus vectors, Ad vectors replicate to very high titers. This makes it possible to efficiently infect all the cells in a population at high MOI to study the consequences of the transient expression of foreign gene products. The recombinant Ads that we have constructed express Py middle T antigen and, in theory, no other Ad gene products after infection of a number of human, simian, and murine cell lines (data not shown). By including the Py enhancer in these viruses, we have been able to increase the level of middle T antigen synthesis in many of these cell lines (unpublished). We have also constructed Ad5-Py recombinants that individually express large and small T antigen and recombinant viruses that express all three T antigens (unpublished). These will be useful to achieve overproduction of the T antigens, to study the individual roles of the T antigens in transformation and tumorigenesis, and to study the transformation of a wide variety of cell strains and lines from a variety of mammals, including humans.

ACKNOWLEDGMENTS

We thank Monica Naujokas for technical assistance, Fiona Lees for typing the manuscript, and Yasha Gluzman for helpful discussions. We also thank Thomas Shenk and John Logan for pMPCV-2 DNA, Steve Dilworth and Beverly Griffin for rat monoclonal antibodies, Ed Harlow for the F4 mouse monoclonal antibody, Bernard Massie and Beatrix Bugler for the peptide antiserum (cT), and R. Treisman and R. Kamen for the middle T-antigen cDNA.

This research was supported by the National Cancer Institute of Canada and the Medical Research Council of Canada. D.D. was the recipient of a Medical Research Council Studentship, and J.A.H. is a research associate and Terry Fox Cancer Research Scientist of the National Cancer Institute of Canada.

LITERATURE CITED

1. **Akusjärvi, G., and H. Persson.** 1980. Controls of RNA splicing and termination in the major late adenovirus transcription unit. Nature (London) **292**:420–426.
2. **Asselin, C., C. Gelinas, and M. Bastin.** 1983. Role of the three polyomavirus early proteins in tumorigenesis. Mol. Cell. Biol. **3**:1451–1459.
3. **Asselin, C., C. Gelinas, P. E. Branton, and M. Bastin.** 1984. Polyomavirus middle T antigen requires cooperation from another gene to express the malignant phenotype in vivo. Mol. Cell. Biol. **4**:755–760.
4. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA **69**:1408–1412.
5. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early

adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12**:721–732.
6. **Berkner, K. L., and P. A. Sharp.** 1983. Generation of adenovirus by transfection of plasmids. Nucleic Acids Res. **11**:6003–6020.
7. **Berkner, K. L., and P. A. Sharp.** 1984. Expression of dihydrofolate reductase, and of the adjacent E1b region, in an Ad5-dihydrofolate reductase recombinant virus. Nucleic Acids Res. **12**:1925–1941.
8. **Berkner, K. L., and P. A. Sharp.** 1985. Effect of the tripartite leader on synthesis of a non-viral protein in an adenovirus 5 recombinant. Nucleic Acids Res. **13**:841–857.
9. **Bolen, J. B., and M. A. Israel.** 1985. Middle tumor antigen of polyomavirus transformation-defective mutant NG59 is associated with pp60$^{c\text{-}src}$. J. Virol. **53**:114–119.
10. **Bolen, J. B., C. J. Thiele, M. A. Israel, W. Yonemoto, L. A. Lipsich, and J. S. Brugge.** 1984. Enhancement of cellular *src* gene product associated tyrosyl kinase activity following polyoma virus infection and transformation. Cell **38**:767–777.
11. **Borrelli, E., R. Hen, and P. Chambon.** 1984. Adenovirus-2 E1a products repress enhancer-induced stimulation of transcription. Nature (London) **312**:608–612.
12. **Bouchard, L., C. Gelinas, C. Asselin, and M. Bastin.** 1984. Tumorigenic activity of polyoma virus and SV40 DNAs in newborn rodents. Virology **135**:53–64.
13. **Carmichael, G. G., and G. K. McMaster.** 1980. The analysis of nucleic acids in gels using glyoxal and acridine orange. Methods Enzymol. **65**:380–391.
14. **Cartwright, C. A., M. A. Hutchinson, and W. Eckhart.** 1985. Structural and functional modification of pp60$^{c\text{-}src}$ associated with polyomavirus middle tumor antigen from infected or transformed cells. Mol. Cell. Biol. **85**:2647–2652.
15. **Cartwright, C. A., P. L. Kaplan, J. A. Cooper, T. Hunter, and W. Eckhart.** 1986. Altered sites of tyrosine phosphorylation in pp60$^{c\text{-}src}$ associated with polyomavirus middle tumor antigen. Mol. Cell. Biol. **6**:1562–1570.
16. **Cogen, B.** 1978. Virus-specific early RNA in 3T6 cells infected by a tsA mutant of polyoma virus. Virology **85**:222–230.
17. **Courtneidge, S. A.** 1985. Activation of the pp60$^{c\text{-}src}$ kinase by middle T antigen binding or by dephosphorylation. EMBO J. **4**:1471–1477.
18. **Courtneidge, S. A., and J. M. Bishop.** 1982. Transit of pp60$^{v\text{-}src}$ to the plasma membrane. Proc. Natl. Acad. Sci. USA **79**:7117–7121.
19. **Courtneidge, S. A., A. D. Levinson, and J. M. Bishop.** 1980. The protein encoded by the transforming gene of avian sarcoma virus (pp60^{src}) and a homologous protein in normal cells (pp60$^{proto\text{-}src}$) are associated with the plasma membrane. Proc. Natl. Acad. Sci. USA **77**:3783–3789.
20. **Courtneidge, S. A., and A. E. Smith.** 1983. Polyoma virus transforming protein associates with the product of the c-*src* cellular gene. Nature (London) **303**:435–439.
21. **Courtneidge, S. A., and A. E. Smith.** 1984. The complex of polyoma virus middle-T antigen and pp60$^{c\text{-}src}$. EMBO J. **3**:585–591.
22. **Dilworth, S. M., and B. E. Griffin.** 1982. Monoclonal antibodies against polyoma virus tumor antigens. Proc. Natl. Acad. Sci. USA **79**:1059–1063.
23. **Eckhart, W., M. A. Hutchinson, and T. Hunter.** 1979. An activity phosphorylating tyrosine in polyoma T antigen immunoprecipitates. Cell **18**:925–933.
24. **Favaloro, J., R. Treisman, and R. Kamen.** 1980. Transcription maps of polyoma virus-specific RNA: analysis by two-dimensional nuclease S1 gel mapping. Methods Enzymol. **65**:718–749.
25. **Featherstone, M. S., M. A. Naujokas, B. J. Pomerantz, and J. A. Hassell.** 1984. A plasmid vehicle suitable for the molecular cloning and characterization of mammalian promoters. Nucleic Acids Res. **12**:7235–7249.
26. **Francke, B., and W. Eckhart.** 1973. Polyoma gene function required for viral DNA synthesis. Virology **55**:127–135.
27. **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.
28. **Garcea, R. L., and T. L. Benjamin.** 1983. Host range transformation gene of polyoma virus plays a role in virus assembly. Proc. Natl. Acad. Sci. USA **80**:3613–3617.
29. **Gluzman, Y., H. Reichl, and D. Solnick.** 1982. Helper-free adenovirus type 5 vectors, p. 187–192. *In* Y. Gluzman (ed.), Eukaryotic viral vectors. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
31. **Grussenmeyer, T., K. H. Scheidtmann, M. A. Hutchinson, W. Eckhart, and G. Walter.** 1985. Complexes of polyoma virus medium T antigen and cellular proteins. Proc. Natl. Acad. Sci. USA **82**:7952–7954.
32. **Heiser, W. C., and W. Eckhart.** 1982. Polyomavirus early and late mRNAs in productively infected mouse 3T6 cells. J. Virol. **44**:175–188.
33. **Higgs, D. R., S. E. Y. Goodbourn, J. Lamb, J. B. Clegg, D. J. Weatherall, and N. J. Proudfoot.** 1983. α-Thalassaemia caused by a polyadenylation signal mutation. Nature (London) **306**:398–400.
34. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
35. **Ito, K., P. J. Bassford, Jr., and J. Beckwith.** 1981. Protein localization in *E. coli*: is there a common step in the secretion of periplasmic and outer-membrane proteins? Cell **24**:707–717.
36. **Ito, Y.** 1979. Polyoma virus-specific 55K protein isolated from plasma membrane of productively infected cells is virus-coded and important for cell transformation. Virology **98**:261–266.
37. **Ito, Y., J. R. Brocklehurst, and R. Dulbecco.** 1977. Virus specific proteins in the plasma membrane of cells lytically infected or transformed by polyoma virus. Proc. Natl. Acad. Sci. USA **74**:4666–4670.
38. **Jones, N., and T. Shenk.** 1979. Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. Cell **17**:683–689.
39. **Kamen, R., J. Favaloro, 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**:63–75.
40. **Kaplan, P. L., S. Simon, and W. Eckhart.** 1985. Polyomavirus middle T protein encoded by a retrovirus transforms nonestablished chicken embryo cells. J. Virol. **56**:1023–1026.
41. **Kessler, S. W.** 1976. Cell membrane antigen isolation with the staphylococcal protein A-antibody absorbent. J. Immunol. **117**:1482–1490.
42. **Khoury, G., J. Alwine, N. Goldman, P. Gruss, and G. Jay.** 1980. New chimeric splice junction in adenovirus type 2-simian virus 40 hybrid viral mRNA. J. Virol. **36**:143–151.
43. **Kornbluth, S., F. R. Cross, M. Harbison, and H. Hanafusa.** 1986. Transformation of chicken embryo fibroblasts and tumor induction by the middle T antigen of polyomavirus carried in an avian retroviral vector. Mol. Cell. Biol. **6**:1545–1551.
44. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
45. **Lanoix, J., R. W. Tseng, and N. H. Acheson.** 1986. Duplication of functional polyadenylation signals in polyomavirus DNA does not alter efficiency of polyadenylation or transcription termination. J. Virol. **58**:733–742.
46. **Lewis, J. B., and M. B. Mathews.** 1980. Control of adenovirus early gene expression: a class of immediate early products. Cell **21**:303–313.
47. **Logan, J., and T. Shenk.** 1984. Adenovirus tripartite leader sequence enhances translation of mRNAs late after infection. Proc. Natl. Acad. Sci. USA **81**:3655–3659.
48. **Magnusson, G., M.-G. Nilsson, S. M. Dilworth, and N. Smolar.** 1981. Characterization of polyoma mutants with altered middle and large T-antigens. J. Virol. **39**:673–683.
49. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory,

Cold Spring Harbor, N.Y.
50. **Mansour, S. L., T. Grodzicker, and R. Tjian.** 1985. An adenovirus vector system used to express polyoma virus tumor antigens. Proc. Natl. Acad. Sci. USA **82**:1359–1363.
51. **Mansour, S. L., T. Grodzicker, and R. Tjian.** 1986. Downstream sequences affect transcription initiation from the adenovirus major late promoter. Mol. Cell. Biol. **6**:2684–2694.
52. **Massie, B., Y. Gluzman, and J. A. Hassell.** 1986. Construction of a helper-free recombinant adenovirus that expresses polyomavirus large T antigen. Mol. Cell. Biol. **6**:2872–2883.
53. **McDonnell, M. W., M. N. Simon, and F. W. Studier.** 1977. Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. J. Mol. Biol. **110**:119–146.
54. **McMaster, G. K., and G. G. Carmichael.** 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. USA **74**:4835–4838.
55. **Mes, A.-M., and J. A. Hassell.** 1982. Polyoma viral middle T antigen is required for transformation. J. Virol. **42**:621–629.
56. **Mes-Masson, A.-M., B. Schaffhausen, and J. A. Hassell.** 1984. The major site of tyrosine phosphorylation in polyomavirus middle T antigen is not required for transformation. J. Virol. **52**: 457–464.
57. **Nilsson, S. V., and G. Magnusson.** 1983. T-antigen expression by polyoma mutants with modified RNA splicing. EMBO J. **2**: 2095–2101.
58. **Nilsson, S. V., C. Tyndall, and G. Magnusson.** 1983. Deletion mapping of a short polyoma virus middle T antigen segment important for transformation. J. Virol. **46**:284–287.
59. **Oppermann, H., A. D. Levinson, H. E. Varmus, L. Levintow, and J. M. Bishop.** 1979. Uninfected vertebrate cells contain a protein that is closely related to the product of the avian sarcoma virus transforming gene (*src*). Proc. Natl. Acad. Sci. USA **76**:1804–1808.
60. **Pettersson, U., and J. Sambrook.** 1973. The amount of viral DNA in the genome of cells transformed by adenovirus type 2. J. Mol. Biol. **73**:125–130.
61. **Prasad, I., D. Zouzias, and C. Basilico.** 1976. State of the viral DNA in rat cells transformed by polyoma virus. I. Virus rescue and the presence of nonintegrated viral DNA molecules. J. Virol. **18**:436–444.
62. **Rassoulzadegan, M., A. Cowie, A. Carr, N. Glaichenhaus, R. Kamen, and F. Cuzen.** 1982. The roles of individual polyoma virus early proteins in oncogenic transformation. Nature (London) **300**:713–718.
63. **Rassoulzadegan, M., Z. Naghashfar, A. Cowie, A. Carr, M. Grisoni, R. Kamen, and F. Cuzen.** 1983. Expression of the large T protein of polyoma virus promotes the establishment in culture of "normal" rodent fibroblast cell lines. Proc. Natl. Acad. Sci. USA **80**:4354–4358.
64. **Schaffhausen, B. S.** 1982. Transforming genes and gene products of polyoma and SV40. Crit. Rev. Biochem. **13**:215–286.
65. **Schaffhausen, B. S., and T. L. Benjamin.** 1979. Phosphorylation of polyoma T antigens. Cell **18**:935–946.
66. **Schaffhausen, B. S., and T. L. Benjamin.** 1981. Protein kinase activity associated with polyoma virus middle T antigen. Cold Spring Harbor Conf. Cell Proliferation **8**:1281–1298.
67. **Schaffhausen, B. S., and T. L. Benjamin.** 1981. Comparison of phosphorylation of two polyoma virus middle T antigens in vivo and in vitro. J. Virol. **40**:184–196.
68. **Schaffhausen, B. S., T. L. Benjamin, J. Lodge, D. Kaplan, and T. M. Roberts.** 1985. Expression of polyoma early gene products in *E. coli*. Nucleic Acids Res. **13**:508–519.
69. **Schaffhausen, B. S., H. Dorai, G. Avakere, and T. L. Benjamin.** 1982. Polyomavirus middle T antigen: relationship to cell membranes and apparent lack of ATP-binding activity. Mol. Cell. Biol. **2**:1187–1198.
70. **Sefton, B. M., T. Hunter, K. Beemon, and W. Eckhart.** 1980. Evidence that the phosphorylation of tyrosine is essential for cellular transformation by Rous sarcoma virus. Cell **20**:807–816.
71. **Segawa, K., and Y. Ito.** 1982. Referential subcellular localization of *in vivo*-phosphorylated and nonphosphorylated middle-sized tumor antigen of polyoma virus and its relationship to middle-sized tumor antigen phosphorylating activity *in vitro*. Proc. Natl. Acad. Sci. USA **79**:6812–6816.
72. **Shaw, A. R., and E. B. Ziff.** 1980. Transcripts from the adenovirus-2 major late promoter yield a single family of 3′ coterminal mRNAs during early infection and five families at late times. Cell **22**:905–916.
73. **Smith, A. E., R. Smith, B. Griffin, and M. Fried.** 1979. Protein kinase activity associated with polyoma middle T antigen *in vitro*. Cell **18**:915–924.
74. **Smith, D. H., D. M. Kegler, and E. B. Ziff.** 1985. Vector expression of adenovirus type 5 E1a proteins: evidence for E1a autoregulation. Mol. Cell. Biol. **5**:2684–2696.
75. **Soeda, E., J. R. Arrand, N. Smolar, J. E. Walsh, and D. E. Griffin.** 1980. Coding potential and regulatory signals of the polyoma virus genome. Nature (London) **283**:445–453.
76. **Solnick, D.** 1981. Construction of an adenovirus-SV40 recombinant producing SV40 T antigen from an adenovirus late promoter. Cell **24**:135–143.
77. **Southern, E. M.** 1975. Detection of specific sequences among fragments separated by agarose gel electrophoresis. J. Mol. Biol. **98**:503–515.
78. **Stow, N. D.** 1981. Cloning of a DNA fragment from the left-hand terminus of the adenovirus type 2 genome and its use in site-directed mutagenesis. J. Virol. **37**:171–180.
79. **Templeton, D., S. Simon, and W. Eckhart.** 1986. Truncated forms of the polyomavirus middle T antigen can substitute for the small T antigen in lytic infection. J. Virol. **57**:367–370.
80. **Thomas, P. S.** 1983. Hybridization of denatured RNA transferred or dotted to nitrocellulose paper. Methods Enzymol. **100**:255–266.
81. **Thummel, C., and R. Tjian.** 1981. Expression of SV40 T antigen under control of adenovirus promoters. Cell **23**:825–836.
82. **Thummel, C., R. Tjian, and T. Grodzicker.** 1982. Construction of adenovirus expression vectors by site-directed *in vivo* recombination. J. Mol. Appl. Genet. **1**:435–446.
83. **Thummel, C., R. Tjian, S.-L. Hu, and T. Grodzicker.** 1983. Translational control of SV40 T antigen expressed from the adenovirus late promoter. Cell **33**:455–464.
84. **Tooze, J.** 1981. DNA tumor viruses, part 2 (revised), 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
85. **Treisman, R., U. Novak, J. Favaloro, 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.
86. **Türler, H., and C. Salomon.** 1985. Small and middle T antigens contribute to lytic and abortive polyomavirus infection. J. Virol. **53**:579–586.
87. **Van Doren, K., and Y. Gluzman.** 1984. Efficient transformation of human fibroblasts by adenovirus-simian virus 40 recombinants. Mol. Cell. Biol. **4**:1653–1656.
88. **Wahl, J. M., M. Stern, and J. 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. USA **76**:3683–3687.
89. **Wigler, M., A. Pellicer, S. Silverstein, and R. Axel.** 1978. Biochemical transfer of single-copy eukaryotic genes using total cellular DNA as donor. Cell **14**:725–731.
90. **Yonemoto, W., M. Jarvis-Morar, J. S. Brugge, J. B. Bolen, and M. A. Israel.** 1985. Tyrosine phosphorylation within the aminoterminal domain of $pp60^{c\text{-}src}$ molecules associated with polyoma virus middle-sized tumor antigen. Proc. Natl. Acad. Sci. USA **82**:4568–4572.
91. **Zain, S., J. Sambrook, R. J. Roberts, W. Keller, M. Fried, and A. R. Dunn.** 1979. Nucleotide sequence analysis of the leader segments in a cloned copy of adenovirus 2 fiber mRNA. Cell **16**:851–861.
92. **Zhu, Z., G. M. Veldman, A. Cowie, A. Carr, B. Schaffhausen, and R. Kamen.** 1984. Construction and functional characterization of polyomavirus genomes that separately encode the three early proteins. J. Virol. **51**:170–180.