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Abundant Expression of Polyomavirus Middle T Antigen and Dihydrofolate Reductase in an Adenovirus Recombinant

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A modular gene with a cDNA encoding the polyomavirus middle T antigen positioned behind the adenovirus type 2 major late promoter and tripartite leader was substituted for the E1a region in an adenovirus vector. Permissive human cells infected with this recombinant produce middle T protein at levels as high as those of the most abundant late adenoviral proteins, e.g., hexon or fiber. This level represents at least a 40-fold increase over that observed in a polyomavirus lytic infection of murine cells. Partial proteolytic mapping showed that this protein has the same primary structure as middle T protein produced in polyomavirus-infected murine cells. The adenovirus recombinant-generated middle T protein exhibited in vitro kinase activity, although at an approximately 10-fold-lower specific activity than that of middle T protein from polyomavirus-infected murine cells. Comparison of the expression levels of this middle T antigen-containing adenovirus vector with a similar construction encoding dihydrofolate reductase suggested that the translation efficiency of the inserted gene was dependent upon the proximity of its initiation codon to the tripartite leader. We tested this possibility by comparing three dihydrofolate reductase recombinants among which the spacing between the initiation codon and tripartite leader varied from 188 to 36 nucleotides. The efficiency of expression of dihydrofolate reductase protein dramatically increased as this spacing was reduced.

Expression of genes that encode scarce proteins in an adenovirus vector, in a manner that mimics the high level of synthesis normally observed for virion proteins at late times, should facilitate the purification and study of these proteins. During the late stage of adenovirus infection, viral mRNAs are preferentially transcribed, transported from the nucleus, and translated. The signals responsible for this preferential expression should include sequences in the major late promoter (MLP) and the mature late mRNAs. Most of the late mRNAs possess a common tripartite leader at their 5′ termini which may contain sequences important for efficient translation at late times (4, 12). We, and others, have described recombinants in which foreign genes were inserted downstream of the adenovirus type 2 (Ad2) MLP (7, 8, 17, 21, 23, 38, 43). Many of these constructs included the tripartite leader, which apparently increased the efficiency of translation. However, in no case was the expression of the inserted gene as high as that for other adenovirus late structural proteins. For example, we found that for an adenovirus recombinant containing the mouse dihydrofolate reductase (DHFR) gene that at early times in the late stage of infection, the DHFR gene was expressed at levels equivalent to those for other structural proteins. However at late times, when the fully regulated viral late state was imposed, the DHFR levels were diminished (8).

One candidate protein for which overexpression would be valuable is the polyomavirus (py) middle T antigen (mT). This protein is normally produced at very low levels in lytic or transformed cells. It is one of three proteins encoded by the early region of the py genome and has been implicated in cell transformation. Mutations in the mT gene can decrease or abolish the ability of py to transform (10, 11, 22, 40, 41). Moreover, a cDNA encoding only pymT is capable of morphologically transforming an established rat cell line (44). In other situations, such as in primary cells, pymT as well as large T or small t antigens or both are essential for transformation (2, 25, 26). Immunoprecipitates of pymT have a tyrosine-specific protein kinase activity that phosphorylates mT as well as exogenous protein substrates (9, 15, 28, 37). Since py mutants affected in this kinase activity are also affected in their transforming ability, a relationship may exist between this biochemical activity and the growth phenotype. The kinase activity is not intrinsic to mT (29, 31, 33). Rather, the activity is acquired by association with the tyrosine-specific kinase pp60$^{c\text{-}src}$ (13), the cellular analog of the oncogene product of Rous sarcoma virus. The binding of pymT to pp60$^{c\text{-}src}$ enhances the tyrosine-specific kinase activity (9, 14, 45).

We substituted into the E1a region of an adenovirus recombinant a pymT cDNA segment. This cDNA segment was positioned downstream of the MLP and tripartite leader sequences. At very late times in infection of permissive cells, this recombinant virus stimulated the synthesis of pymT at levels as high as that of other late structural proteins. Differences in expression levels between a DHFR-encoding recombinant described previously (8) and this mT recombinant suggested an inverse relationship between the efficiency of translation and the distance between the tripartite leader and the initiation codon. We tested this possibility by positioning the initiation codon of the DHFR segment closer to the tripartite leader sequences. The level of expression of DHFR in such a recombinant now mimics that of the adenovirus late structural proteins.

MATERIALS AND METHODS

Cells and viruses. Wild-type py NG59RA used for controls was derived from strain NG59 by marker rescue (3, 16).

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Virus stocks were propagated on 3T6 cells grown in Dulbecco modified Eagle medium supplemented with 5% calf serum. The wild-type adenovirus was Ad5(309) (19). Adenovirus stocks were prepared on 293 cells grown in Dulbecco modified Eagle medium containing 5% fetal calf serum.

T-antigen analysis. The detailed procedures for isolation and analysis of T antigens have been published previously (28–30). Infected cells were labeled with [^{35}S]methionine (40 μCi/ml) for 90 min in Hanks solution. Labeled or unlabeled cells were extracted in buffer containing 1% (vol/vol) Nonidet P-40. Cleared extracts were incubated with anti-T ascites fluid and protein A-Sepharose (Pharmacia, Inc.). For the in vitro kinase reactions, washed immunoprecipitates were incubated with 5 to 10 μCi of [γ-^{32}P]ATP in 0.02 M Tris hydrochloride (pH 7.5)–0.005 M $MgCl_2$ for 15 min at room temperature.

Partial proteolytic mapping was carried out by first running labeled proteins on cylindrical discontinuous-buffer sodium dodecyl sulfate gels of 10% acrylamide. The portions of the cylinder containing pymT were placed on top of a 12.5% acrylamide slab gel. Digestion was carried out by overlaying the cylinders with 2 ml of a solution containing 50 μg of bovine serum albumin per ml and either 20 μg of *Staphylococcus aureus* V8 protease (Miles Laboratories, Inc.) per ml or 30 μg of chymotrypsin per ml (Worthington Diagnostics). Electrophoresis was carried out at 50 V until the bromphenol blue marker reached the bottom of the gel.

RNA and protein analyses. RNA preparation and analysis were performed essentially as previously described (6, 7). Multiplicities of infection between 10 and 20 were used, and cells were harvested at early times (8 h) or late times (24 h) in infection. For early-infected RNA preparations, cytosine arabinoside was added to the media (to 20 μg/ml) 1 h postinfection. Nuclease S1 analysis was performed by using end-labeled probes, at the melting temperature (T_m) determined for each, as detailed in the figure legends. The RNA-DNA hybrids were either resolved on neutral 1.4% agarose gels or subjected to denaturation in formamide and electrophoresed through 8% acrylamide (40:1 acrylamide to bisacrylamide)–urea gels (24).

Cells from the 293 cell line were labeled for 1 h with [^{35}S]methionine (25 μCi/ml) in serum-free, methionine-free Dulbecco modified medium at various times after infection. Lysates were prepared and analyzed by electrophoresis in gels composed of either 15% acrylamide (180:1 acrylamide to bisacrylamide) for analysis of DHFR protein, or 10% acrylamide (38:1 acrylamide to bisacrylamide) for analysis of pymT.

Plasmid and virus constructions. pAd5(pymT) was constructed from pDHFRIII (8) by first partially digesting pDHFRIII with *Pst*I to modify the *Pst*I site which adjoins the DHFR cDNA to the expression vector (Fig. 1). The *Pst*I termini were rendered blunt by incubation with T4 DNA polymerase and dCTP, and *Bam*HI linkers were then ligated to the termini. After cleavage with *Bam*HI, the new *Bam*HI site was joined to the natural *Bam*HI site in pDHFRIII (Fig. 1), thereby generating the expression vector lacking the simian virus 40 (SV40) early polyadenylation signal, pD(ΔpA).

The pymT cDNA (1.8 kilobases [kb]) was isolated as a *Bcl*I-*Bam*HI fragment from pT890 (31) and ligated to a gel-purified 0.2-kb *Bcl*I-*Bam*HI fragment from SV40 DNA. The DNA was subsequently cleaved with *Bam*HI and *Bcl*I, and the appropriate 2.0-kb ligation-cleavage product was gel purified. This fragment was inserted into the *Bam*HI site of pD(ΔpA) to generate pAd5(pymT). All plasmid constructions were verified for orientation and structure by restriction endonuclease analysis. The plasmid pAd5(pymT) was adjoined to viral sequences (4 to 100 map units [m.u.]) from strain 309 DNA, as described previously (39), via the *Xba*I site. The DNA was transfected onto 293 cells, and subsequent virus stocks were purified by double-plaque isolation.

To delete 89 of the bases between the *Pst*I site and the initiation codon in pDHFRIII, the *Pst*I site immediately upstream of the DHFR cDNA was first converted to a *Bcl*I site, essentially as described above and as outlined in Fig. 6. *Bcl*I-linkered DNA was cleaved with *Bcl*I and *Nco*I, and the 5.3-kb fragment was gel purified. The pDHFRIII plasmid was also cleaved with *Fnu*4HI, which generates approximately 20 fragments. This was followed by incubation with T4 DNA polymerase and all four deoxynucleoside triphosphates to generate blunt termini. The ends were ligated to *Bam*HI linkers, and *Bam*HI-*Nco*I digestion was carried out. The 0.6-kb *Bam*HI-*Nco*I fragment containing the DHFR cDNA (ΔDHFR) was isolated and subsequently ligated to the 5.3-kb *Bcl*I-*Nco*I fragment (see Fig. 6) to generate pΔDHFRIII. This DNA was used to prepare adenovirus recombinant stocks, as described for the pymT construction. The sequences upstream of the initiation codon of pΔDHFRIII were confirmed by sequencing (24), after the DNA was end labeled at the *Bgl*II site (see Fig. 6).

A fragment of 152 bp, which did not contain any ATGs, was inserted into the deleted region of pΔDHFRIII. An *Mbo*I (2,409 bp)-to-*Pst*I (2,257 bp) digestion fragment from pUC13 DNA was isolated by gel electrophoresis. This fragment was ligated to the 5.3-kb *Pst*I-*Nco*I fragment from pDHFRIII, which contains most of the vector, and to the 0.6-kb *Bam*HI-*Nco*I fragment from pΔDHFRIII, which con-

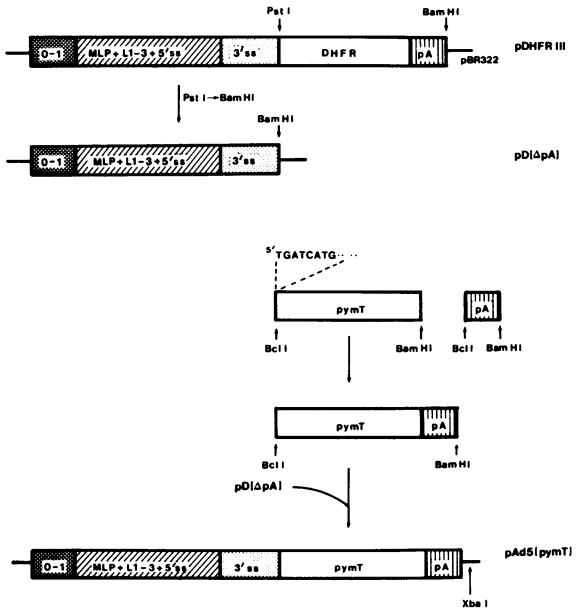

FIG. 1. Construction of pAd5(pymT). The details of the construction of plasmids pD(ΔpA) and pAd5(pymT) are described in Materials and Methods. The pymT cDNA is 1.8 kb in length, and the nucleotides shown above the pymT cDNA form the 5′ terminus. The thin line in all cases represents pBR322 sequences. The *Xba*I site indicated for pAd5(pymT) was generated by linkering the *Sal*I site in pBR322 DNA and is the site of ligation with Ad5(309) (4 to 100 m.u.) DNA used in producing the viral recombinants.

tains ΔDHFR (see Fig. 6). Plasmid DNA of this product was analyzed by restriction endonuclease digestion to verify the correct structure. It was then further modified by elimination of an *Xba*I site from the inserted sequences: after partial digestion with *Xba*I, the termini were incubated with T4 DNA polymerase and all four deoxynucleoside triphosphates and then ligated with T4 DNA ligase. The sequences modified in generating this recombinant, pinsDHFRIII, from pΔDHFRIII were verified by Maxam-Gilbert sequencing. Viral recombinants were subsequently generated by transfection with pinsDHFRIII, as described above.

Materials. All radioisotopes were obtained from New England Nuclear Corp. Restriction endonucleases, T4 DNA kinase, T4 DNA ligase, and T4 DNA polymerase were obtained from New England BioLabs, Inc., or Bethesda Research Laboratories, Inc. The S1 nuclease was purchased from Miles.

RESULTS

Construction of an adenovirus vector expressing pymT. A cDNA segment encoding pymT was inserted into the modular adenovirus vector described previously (Fig. 1) (8). In this vector, the cDNA segment was positioned downstream of sequences encoding the Ad5 left terminus (0 to 1 m.u.), the Ad2 MLP, a cDNA fragment encoding the entire Ad2 tripartite leader, and a set of splice signals derived from the third leader 5′ splice site and a 3′ splice site from an immunoglobulin gene. A mature mRNA expressed from this transcription unit should have an identical tripartite leader to that of other late mRNAs. The 3′ end of the pymT cDNA segment was adjoined to a fragment containing the early polyadenylation signal of SV40. This modular expression unit was first formed in a plasmid, pAd5(pymT), which was subsequently cleaved at the *Xba*I site and ligated to a purified 4- to 100-m.u. fragment from Ad5(309) viral DNA (39). This ligation product was transfected onto 293 cells, and a recombinant virus was subsequently plaque purified.

Stocks of the viral recombinant, Ad5(pymT), were found to be approximately 100-fold lower in titer than Ad5(309), although the growth characteristics of both types of virus were otherwise similar on 293 cells. The lower yields of recombinant virus appeared to be a consequence of poor packaging of the recombinant DNA, presumably owing to its large genomic size (106 m.u.). When Ad5(pymT) virus stocks were coinfected with another recombinant virus, Ad5(DHFRIII), which is 103 m.u. in length and which yields wild-type titers (8), equal amounts of DNA synthesis for each virus were observed at late times (20 to 40 h) in infection. The titer of Ad5(DHFRIII) from this coinfection was identical to that observed after infection with Ad5(DHFRIII) alone. Thus, expression of the inserted gene in Ad5(pymT) did not suppress in *trans* the growth of another adenovirus recombinant.

pymT is abundantly produced during Ad5(pymT) infection and is phosphorylated in an in vitro kinase reaction. When 293 cells were infected with Ad5(pymT), mRNAs from the modular transcriptional unit were expressed at levels comparable to those of late mRNAs encoding virion polypeptides, such as fiber mRNA (data not shown). The levels were also approximately the same as that from a similar Ad5 recombinant containing a mouse DHFR cDNA gene, Ad5(DHFRIII) (8; also described below). High levels of mRNA synthesis had previously been demonstrated for this recombinant. RNA complementary to pymT sequences was detected only late in infection, and both the spliced and unspliced forms of the mRNA were observed, at a ratio of approximately 4:1. The spliced mRNA was the predicted size if the signals built into the modular transcriptional expression unit (Fig. 1) were used appropriately (data not shown).

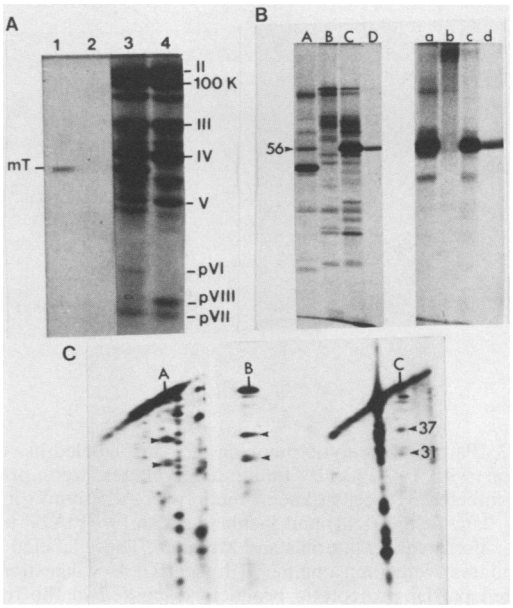

FIG. 2. High-level expression of pymT in Ad5(pymT)-late-infected 293 cells. (A) 293 cells were infected (multiplicity of infection, 10), and 20 h later, newly synthesized proteins were labeled with [^{35}S]methionine for 1 h. Immunoprecipitates and lysates of Ad5(309) (lanes 2 and 4, respectively) and Ad5(pymT) (lanes 1 and 3, respectively) were resolved on an acrylamide gel, as detailed in Materials and Methods. (B) Equal numbers of cells (293 or 3T6) were labeled in vivo with [^{35}S]methionine, as described in Materials and Methods for T-antigen analysis. This was followed by Nonidet P-40 extraction and immunoprecipitation with monoclonal antibodies specific for pymT. Aliquots of the immunoprecipitates were either directly analyzed by electrophoresis through acrylamide (lanes A to D) or tested for in vitro kinase activity by incubation with [γ-^{32}P]ATP before analysis by gel electrophoresis (lanes a to d). Immunoprecipitates from py-infected 3T6 cells (lanes A, a), mock-infected 293 cells (lanes B, b), Ad5(pymT)-infected 293 cells (lanes C, c), and 1/10 of this sample (lanes D, d) were compared. (C) In vivo [^{35}S]methionine-labeled protein from Ad5(pymT)-infected 293 cell extracts (A), or immunoprecipitated extracts from Ad5(pymT)-infected 293 cells (B) or py-infected 3T6 cells (C) were analyzed by partial chymotryptic digestion, as described in Materials and Methods. Arrows denote the 37- and 31-kDa fragments of pymT.

High levels of mT mRNA in 293 cells infected by Ad5(pymT) resulted in correspondingly high levels of synthesis of mT protein. When total cell protein was extracted from cells labeled with [^{35}S]methionine, a 56-kilodalton (kDa) polypeptide (the 56K polypeptide) was observed in Ad5(pymT)- but not in Ad5(309)-infected cells (Fig. 2A). This labeled polypeptide was as abundant as two of the prominently produced viral polypeptides, hexon and fiber. The 56-kDa band was identified as pymT by its immunoprecipitation from extracts of infected cells with monoclonal antibodies specific for pymT (Fig. 2A, lane 1) and by partial proteolytic digestion (Fig. 2C). Chymotryptic digestion of the 56-kDa form of pymT antigen gives rise to characteristic fragments of 37 and 31 kDa (29, 30). The same fragments were generated by digestion of the mT protein synthesized after infection of 293 cells with Ad5(pymT). Figure 2C,

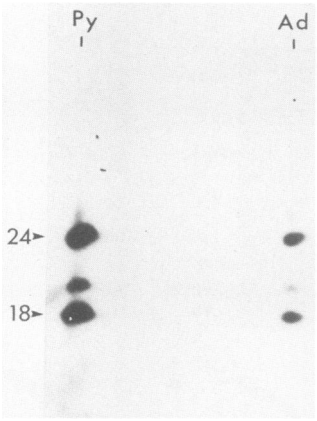

FIG. 3. Partial proteolytic mapping of pymT labeled in vitro by incubation with [γ-^{32}P]ATP. Immunoprecipitates were prepared from py-infected 3T6 cells (lane at the left) or Ad5(pymT)-infected 293 cells (lane at the right) and incubated with [γ-^{32}P]ATP to label pymT as described in Materials and Methods. These labeled immunoprecipitates were then compared by partial V-8 digestion. The anticipated partial proteolytic products discussed in the text are indicated by arrows and molecular weights (expressed in thousands).

shows a parallel analysis of the [^{35}S]methionine-labeled chymotryptic products of an extract of Ad5(pymT)-infected cells with an immunoprecipitated sample from py-infected 3T6 cells. mT polypeptides from these two sources generated comigrating 37- and 31-kDa fragments. Chymotryptic digestion of the 56K protein immunoprecipitated from Ad5(pymT)-infected 293 cells also yielded the same two fragments.

Expression of mT in 293 cells infected with Ad5(pymT) was also compared directly with that during a py lytic infection of mouse 3T6 cells (Fig. 2B). The adenovirus recombinant infection yielded at least a 40-fold-higher level of pymT polypeptide than did the py infection.

Immunoprecipitates of pymT from either infected or transformed cells have been shown to possess a tyrosine kinase activity, owing to the binding of the cellular pp60$^{c\text{-}src}$ protein (9, 13), which phosphorylates the mT polypeptide (15, 29, 35, 37). To test whether a similar kinase activity was associated with pymT synthesized after infection of 293 cells with Ad5(pymT), extracts of infected cells were immunoprecipitated with a monoclonal antibody to pymT and incubated with [γ-^{32}P]ATP in an in vitro kinase assay. Incubation of the immunoprecipitate from Ad5(pymT)-infected cells produced a heavily phosphorylated 56-kDa band (Fig. 2B, lane c), while the equivalent reaction from the control Ad5(309)-infected cells was negative (Fig. 2B, lane b). An assay of the immunoprecipitate from py-infected 3T6 cells yielded a comigrating ^{32}P-labeled mT band (Fig. 2B, lane a). Thus a kinase activity is associated with pymT when synthesized in human cells. The kinase specific activity of mT immunoprecipitated from py-infected 3T6 cells was at least 10-fold higher than that immunoprecipitated from Ad5(pymT)-infected 293 cells. This was observed in repeated experiments in which a 40-fold-higher level of mT was immunoprecipitated from Ad5(pymT)-infected than from py-infected cells, yet the relative amount of kinase activity in the two immunoprecipitates varied only between one- and fourfold (Fig. 2B).

Previous work has shown that the in vitro activity associated with mT in mouse cells is specific for tyrosine residues (15). In py-infected 3T6 cells, the principal site of phosphorylation is tyrosine 315, and tyrosine residues 322 and 250 can be modified as well (15, 29). The Ad5(pymT) protein is probably similarly phosphorylated (Fig. 3). *S. aureus* V8 protease digestion of pymT generates 24- and 18-kDa fragments, which represent overlapping C-terminal peptides containing both tyrosine 315 and 322 phosphorylation sites. The V8 protease digestion profile of in vitro-labeled Ad5(pymT) protein was identical to that derived from py-infected 3T6 cells (Fig. 3).

Signals required for efficient translation at late times in infection. The efficient synthesis of pymT at late times after infection of 293 cells with Ad5(pymT) was surprising, given previous observations with a similar recombinant, Ad5(DHFRIII) (8). This latter adenovirus recombinant was constructed by insertion of a cDNA segment for the mouse DHFR gene into the equivalent position as the cDNA segment encoding pymT in Ad5(pymT). At late times after infection of 293 cells with Ad5(DHFRIII), high levels of mature mRNAs were expressed from the modular gene, but the efficiency of translation of these mRNAs was low (8). This result is duplicated in Fig. 4, in which the levels of synthesis of either pymT or DHFR polypeptides were compared at various times after infection with Ad5(pymT) or Ad5(DHFRIII), respectively. Both recombinant viruses stimulated synthesis of high levels of the appropriate polypeptide at 12 and 18 h postinfection. However, by 24 h postinfection, the rate of synthesis of DHFR polypeptide dropped dramatically, while the rate of synthesis of pymT polypeptide remained high. Thus, at very late times, the mRNA encoding pymT could compete efficiently for translation with late mRNAs encoding virion components.

A number of alternatives might account for the differential expression of pymT and DHFR genes. Although both cDNAs were constructed in the same modular expression vehicle, insufficient fine structural analysis had been done to exclude nonidentical splice sites or polyadenylation usage. To determine whether the mRNAs encoding either pymT or DHFR utilized the same splice sites and polyadenylation signal, mRNAs from infected cells were analyzed by the S1 nuclease methodology (Fig. 5). Using probes with labeled 3′

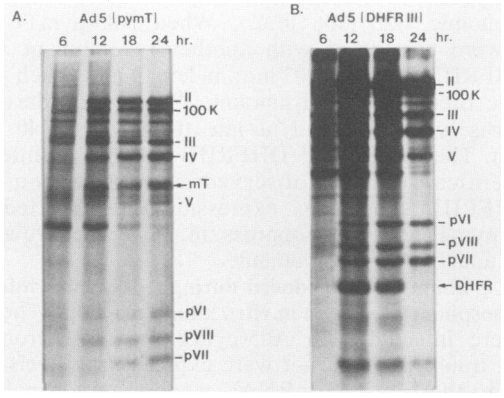

FIG. 4. Comparison of the temporal expression of two different adenovirus recombinants. 293 Cells infected with adenovirus recombinants (multiplicity of infection, 20) encoding pymT (A) or DHFR (B) were labeled with [^{35}S]methionine for 1 h in vivo, at the postinfection times indicated. Extracts of these cells were then analyzed by acrylamide gel electrophoresis as described in Materials and Methods. The mobility of the pymT and DHFR polypeptides are indicated.

termini for S1 analysis, we showed that the two transcripts terminated at the SV40 polyadenylation signal (Fig. 5A). Moreover, both pymT mRNA and DHFR mRNA were generated by splicing between the third leader 5′ splice site (data not shown) and the immunoglobulin 3′ splice site (Fig. 5B).

Among those late mRNAs that have been analyzed, the number of nucleotides which separate the tripartite leader and initiation codon is small. This suggests that the inefficient translation of DHFR mRNA could have been due to the 125 nucleotides of separation in mRNA from this recombinant as compared with the 37 nucleotides of separation in mRNAs from the Ad5(pymT) recombinant. To test this hypothesis, part of the 5′ noncoding sequence in the DHFR recombinant was deleted. The DHFR cDNA segment in the plasmid pDHFRIII was digested with the restriction endonuclease *Fnu*4HI, which cleaves 7 nucleotides upstream of the initiation codon. This terminus was joined to the virus vector by ligation of *Bam*HI linker oligonucleotides (Fig. 6).

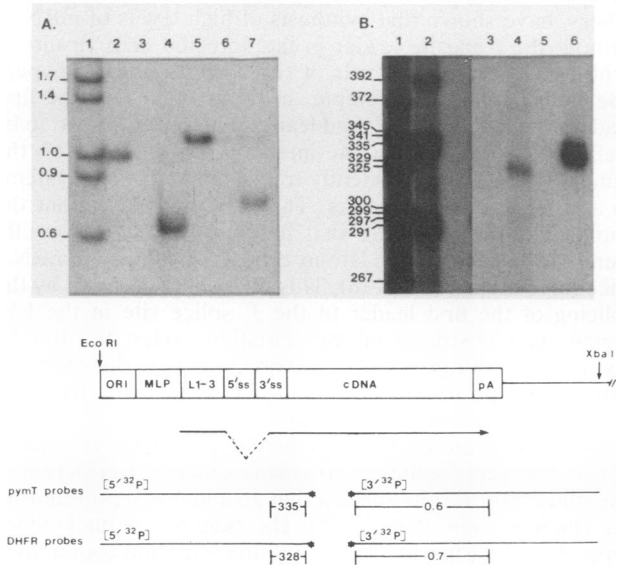

FIG. 5. Comparison of the RNA structure from Ad5(pymT)- and Ad5(DHFRIII)-infected 293 cells. (A) *Pst*I-cleaved pymT cDNA and *Aha*III-cleaved DHFR cDNA were 3′ end labeled with T4 DNA polymerase and α-^{32}P-labeled deoxynucleoside triphosphates. These probes were also cleaved with *Xba*I and then used to analyze the 3′ termini by the S1 nuclease method (5). Hybridization temperatures of 50°C (DHFR) and 52°C (pymT) were used. The samples were electrophoresed through a 1.4% native agarose gel. Lanes: 1, *Eco*RI-*Hin*dIII digestions of λDNA as markers; 2, pymT probe alone; 3, pymT probe with Ad(pymT) early mRNA; 4, pymT probe with Ad5(pymT) late mRNA; 5, DHFR probe alone; 6, DHFR probe with Ad5(DHFRIII) early mRNA; 7, DHFR probe with Ad5(DHFRIII) late mRNA. (B) *Eco*RI-cleaved pymT cDNA and *Sst*I-cleaved DHFR cDNA were 5′ end labeled with T4 polynucleotide kinase. The DHFR cDNA was subsequently cleaved with *Eco*RI. The probes were hybridized to RNA at 50°C, and then digested with S1 nuclease. The protected DNA fragments were analyzed by electrophoresis in an acrylamide gel under denaturing conditions, as described in Materials and Methods. Lanes: 1, *Hae*III digestions of SV40 and pBR322 DNA as markers; 2, *Hae*III digestion of φX174 DNA as markers; 3, DHFR probe with early DHFR mRNA; 4, DHFR probe with late DHFR mRNA; 5, pymT probe with early pymT mRNA; 6, pymT probe with late pymT mRNA. The anticipated lengths of digestion products are indicated in the diagrams at the bottom. Unspliced mRNAs were also observed with both pymT mRNA and DHFR mRNA (not shown above).

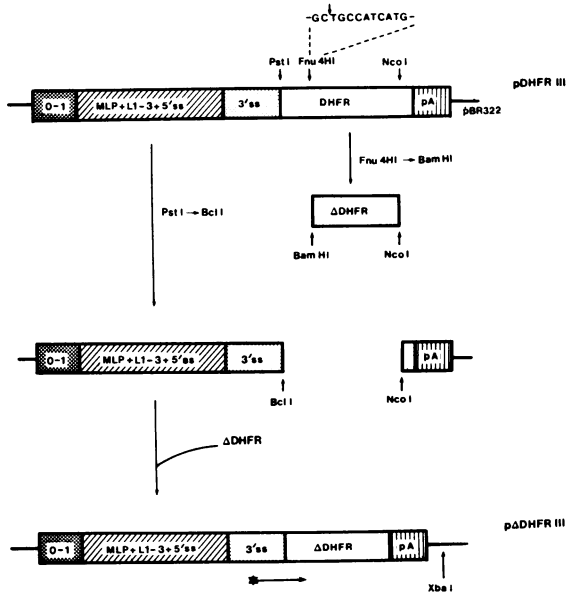

FIG. 6. Construction of a DHFR cDNA truncated in the 5′ noncoding sequences. Two derivatives of the pDHFRIII plasmid were prepared in which the indicated *Fnu*4HI site was converted to a *Bam*HI site and the indicated *Pst*I site was converted to a *Bcl*I site. The details for these steps are given in Materials and Methods. To ensure that the modifications were as expected, approximately 200 bases of pΔDHFRIII were sequenced from the *Bgl*II site (indicated by the asterisk). pΔDHFRIII was found by both restriction enzyme analysis and sequencing to contain an extra *Bam*HI linker at the 5′ terminus of the DHFR cDNA. This modification deletes 89 bases between the ATG codon of the DHFR cDNA and the 3′ splice site. The *Xba*I site in pΔDHFRIII was adjoined to viral sequences, as outlined in the legend to Fig. 1.

The ligation junction of the final construct, p(ΔDHFRIII), was sequenced. This plasmid was incorporated into a recombinant virus stock, Ad5(ΔDHFRIII), as described above. Ad5(ΔDHFRIII) should generate mature mRNA with 36 nucleotides separating the tripartite leader and the initiation codon of DHFR.

When 293 cells were infected with Ad5(DHFRIII) and Ad5(ΔDHFRIII), equal amounts of mRNA were observed by both Northern analysis and S1 nuclease analysis (data not shown). The structure of these mRNAs was shown to differ only by the sequences deleted in Ad5(ΔDHFRIII). DHFR polypeptide was expressed at high levels at early times late after infection with either recombinant virus stock (Fig. 4 and 7). However, expression of DHFR polypeptide by the two viruses was distinguishable at late times in infection (24 h): while DHFR protein synthesis decreased in Ad5(DHFRIII) infection, the levels of DHFR protein synthesized after Ad5(ΔDHFRIII) infection remained high. These high levels were equivalent to that of the other late Ad5 structural proteins (Fig. 7). Thus, shortening the spacing between the tripartite leader and initiation codon of DHFR increased the efficiency of translation of the mRNA at late times in infection.

If the difference in the efficiency of translation of DHFR mRNA between Ad5(DHFRIII) and Ad5(ΔDHFRIII) infection was due only to the spacing between the tripartite leader and the initiation codon and not specifically to the unique sequence deleted from Ad5(DHFRIII), then insertion of other spacer sequences into the same site in Ad5(ΔDHFRIII)

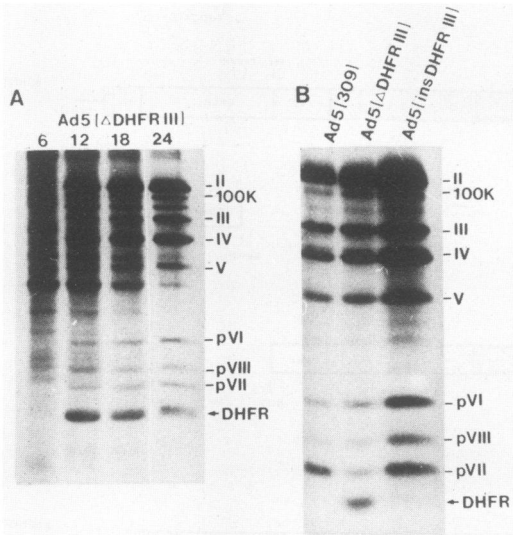

FIG. 7. Expression of Ad5(ΔDHFRIII) and Ad5(insDHFRIII). 293 cells infected at a moi of 10 were labeled with [^{35}S]methionine in vivo for 1 h at the indicated times postinfection (A) or at 24 h postinfection (B). Lysates were then prepared and analyzed by acrylamide gel electrophoresis, as detailed in Materials and Methods. The mobility of the DHFR polypeptide is indicated.

should decrease the efficiency of DHFR synthesis. To test this prediction, we inserted a 152-base-pair fragment which lacked any methionine codons into the Ad5(ΔDHFRIII) recombinant. A fully processed mRNA encoding DHFR transcribed from this recombinant Ad5(insDHFRIII) should contain 188 nucleotides between the tripartite leader and the initiation codon. When parallel cultures of 293 cells were infected with Ad5(ΔDHFRIII) and Ad5(insDHFRIII) and pulse-labeled with [^{35}S]methionine 24 h later, the level of DHFR protein synthesis observed in the Ad5(insDHFRIII) infection was much lower (Fig. 7). This level approximated that observed with Ad5(DHFRIII) (compare Fig. 4 and 7). Hence, we conclude that short lengths of separation between the tripartite leader and the initiation codon are important for efficient translation of mRNAs at late stages of infection.

DISCUSSION

By modifying a previously described adenovirus vector (8), we generated a virus recombinant which can express inserted genes at levels comparable to those for the most abundant late viral polypeptides. This represents one of the most potent systems yet developed for obtaining abundant protein production. The modular transcription unit in the vector is composed of the Ad2 MLP, a cDNA segment encoding the entire tripartite leader, the third leader 5′ splice site, a 3′ splice site from an immunoglobulin gene, and a segment containing the early polyadenylation signal of SV40. This modular transcriptional unit was substituted for the E1a region in the recombinant so that viral stocks can be replicated to high titers by infection of 293 cells, a permissive human cell line containing integrated copies of the E1a region (18). The ability to generate homogeneous virus preparations is advantageous for obtaining maximal expression of any foreign gene inserted into the recombinant. Two cDNA segments have been inserted into this adenovirus vector and expressed at high levels: one encoding the pymT in recombinant virus Ad5(pymT) and the other encoding mouse DHFR in recombinant virus Ad5(ΔDHFRIII).

Synthesis of mRNA from the recombinant transcriptional unit was restricted to the late stages of infection (7, 8; Fig. 5). At this stage, the abundance of mRNA from the modular unit is comparable to that of late mRNAs encoding abundant structural polypeptides such as fiber and the 100K polypeptide. The inability to detect mRNA from the recombinant unit at early times in infection contrasts with previous reports of a low level of transcription from the natural MLP at early stages of (wild-type Ad5) infection (20). The segment containing the major late promoter in the recombinant unit encompassed only 200 base pairs upstream of the TATAA sequences (Fig. 1). This segment may not contain sequences specifying transcription during the early stage.

During the late stages of infection, adenovirus mRNAs are selectively translated and must therefore contain unique signals that effect this preferential recognition. Most late adenovirus mRNAs have the tripartite leader segments spliced to their 5′ termini, and this common element might contain the signals for preferential translation. We, and others, have shown that synthesis of high levels of mRNAs without the tripartite leader in late-infected cells results in synthesis of only low levels of the corresponding polypeptide (7, 38, 43). For example, mRNAs with only the first leader, or the first and second leader, have been shown to be inefficiently translated at late-infection times. However, the same mRNAs are as efficiently translated in in vitro systems as are the late viral mRNAs. The only mRNA without the complete tripartite leader that has been shown to be efficiently translated during late infection is an unusual mRNA encoding polypeptide IX (8). This mRNA was formed by the splicing of the first leader to the 3′ splice site in the E1b region, just upstream of the initiation codon for the IX polypeptide. Mature mRNAs expressed from the recombinant transcription unit described above have the tripartite leader spliced to their 5′ termini.

Synthesis of an mRNA with the complete tripartite leader is not, however, sufficient to ensure efficient translation at late times. Our results suggest that an additional prerequisite for efficient translation is that the number of nucleotides separating the tripartite leader and the initiation codon must also be small. We have previously described an adenovirus recombinant, Ad5(DHFRIII), which contained cDNA sequences encoding the mouse DHFR protein in an equivalent modular gene construction to Ad5(pymT) (8). While infection of 293 cells with the Ad5(pymT) recombinant yielded high levels of translation of pymT at all times during late infection, DHFRIII mRNA was efficiently translated at the beginning of the late stage of infection but was not efficiently translated during the late part of the late stage (24 h; Fig. 4). We had suggested that this decrease in levels of synthesis of DHFR polypeptide at late times was due to the inability of the DHFRIII mRNA to compete with the other mRNAs that accumulate at this time (8). Obviously, the mRNA encoding pymT competed efficiently for translation at late times. In the mRNA encoding pymT, the tripartite leader was 37 nucleotides upstream of the initiation codon, whereas Ad5(DHFRIII) specified the synthesis of an mRNA with the tripartite leader 125 nucleotides upstream of the initiation codon. To test the hypothesis that the length of this separation was important, 89 nucleotides were deleted from the region between the tripartite leader and the initiation codon in the latter construct, generating a similar adenovirus recombinant, Ad5(ΔDHFRIII). After infection of 293 cells with Ad5(ΔDHFRIII), the level of DHFR protein synthesis

remained high during all times of the late stage. As a further test, we have shown that this level could be reduced dramatically by inserting a different (152-base-pair) sequence into Ad5(ΔDHFRIII) between the tripartite leader and the initiation codon. Thus it is likely that the spacing between the tripartite leader and the initiation codon, rather than specific sequence content, is important for translation efficiency. The distance of 36 nucleotides between the tripartite leader and initiation codon in Ad5(ΔDHFRIII) mRNA falls within the range observed for late viral mRNAs, e.g., fiber and hexon mRNAs have separations of 0 and 38 nucleotides, respectively (1, 46).

Some combination of viral and cellular factors must effect selective translation of mRNA with a tripartite leader at late times. Thimmappaya et al. (42) have shown that the small virus-associated RNA, V.A. RNA I, is essential for translation at late stages of infection. An adenovirus mutant defective for synthesis of V.A. RNA I fails to translate mRNAs at late times, including late mRNAs that do not contain a tripartite leader as well as normal cellular mRNAs. Cells infected with these mutants have a generalized block in polypeptide chain initiation (27, 34, 36) at late times. Thus V.A. RNA I probably does not specify the selective translation of mRNAs containing a tripartite leader observed in late stages of infection.

The level of pymT synthesis observed after infection of 293 cells with Ad5(pymT) was comparable to those of the most abundant late-virion polypeptides, e.g., hexon and fiber. The mT accumulated to levels of approximately 10 mg/liter, as determined both by Coomassie staining of gels of infected cell lysates (not shown) and by the amounts recovered after protein purification (32). This level was at least 40-fold higher than that produced after py infection of permissive 3T6 cells. The mT synthesized in adenovirus recombinant-infected cells was identified by immunoprecipitation with monoclonal antibodies specific for pymT and by comparison of proteolytic cleavage products. Immunoprecipitates of mT from adenovirus recombinant-infected human cells possessed an in vitro kinase activity that phosphorylated the mT. This phosphorylation occurred on the same sites that are modified in immunoprecipitates of mT from py-infected cells (Fig. 3) (29, 30). Comparison of the specific kinase activity, i.e., the amount of phosphorylation of mT per amount of mT immunoprecipitated from cells, suggests that the specific kinase activity from Ad5(pymT)-infected 293 cells was 1/10 or less that from py-infected 3T6 cells. Thus, the high levels of synthesis of mT in the adenovirus-infected cells did not yield corresponding increases in the kinase activity associated with mT. A similar result has been observed previously with an SV40 vector system (47). Considerable evidence now suggests that the kinase activity arises from $pp60^{c\text{-}src}$, which is associated with a small fraction of the mT molecules (9, 13). The lower specific kinase activity in Ad5(pymT)-infected cells presumably results from limitations in the ability of the abundant mT protein to associate with human $pp60^{c\text{-}src}$ (32).

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