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Adenovirus Preterminal Protein Synthesized in COS Cells from Cloned DNA Is Active in DNA Replication In Vitro

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Replication of the DNA genome of human adenovirus serotype 2 requires three virus-encoded proteins. Two of these proteins, the preterminal protein (pTP) and the adenovirus DNA polymerase, are transcribed from a single promoter at early times after virus infection. The mRNAs for these proteins share several exons, including one encoded near adenovirus genome coordinate 39. By using plasmids containing DNA fragments postulated to encode the various exons of pTP mRNA, the contributions of each exon to the synthesis of an active pTP have been measured. Only plasmids that contain both the open reading frame for pTP (genome coordinates 29.4 to 23.9) and the *Hin*dIII J fragment that contains the exon at genome coordinate 39 can express functional pTP.

A soluble adenovirus DNA replication system that maintains the fidelity of the intracellular process has been developed. This replication system has facilitated the characterization of the requirements for viral DNA synthesis (2, 19, 23–25, 31). This process is dependent on the addition of exogenous viral DNA covalently linked to a 55-kilodalton (kDa) terminal protein (4, 31), as well as three virus-encoded polypeptides: the 59-kDa single-stranded DNA binding protein (DBP) (9, 19), the preterminal protein (pTP) (3, 23, 32), and the adenovirus DNA polymerase (AdPol) (10, 24, 25, 33). The adenoviruses use a protein-priming mechanism to initiate DNA replication from identical origin sequences included in inverted repetitions found at each end of the linear double-stranded virus DNA (for reviews, see references 11, 22, and 34). In addition to the virus polypeptides, at least three factors present in uninfected nuclear extracts are required (26–28). Two of these host proteins, factors I and III, are site-specific DNA-binding proteins that recognize sequences within the first 50 base pairs (bp) at the ends of the viral DNA. The third host polypeptide required is factor II, a type I topoisomerase. For the initiation of adenovirus DNA replication, the covalent attachment of the first deoxyribonucleotide, dCMP, to pTP requires AdPol, pTP, adenovirus DNA with the 55-kDa terminal protein covalently linked to each 5′ end (AdDNA-pro), and host factors I and III. Elongation beyond the first base also requires the viral DBP (9), whereas factor II (the topoisomerase I activity) is required when the replication fork has moved beyond 20% of the length of the viral genome.

The three viral gene products are transcribed from the same promoter at genome coordinate 75 at early times after infection; however, their subsequent pattern of RNA splicing differs considerably (32). The mRNAs that encode the pTP and AdPol polypeptides have been mapped by electron microscopy and have been shown to contain several noncontiguous exons from the adenovirus genome. In addition to a leader segment near the promoter for these mRNAs at genome coordinate 75, there are three exons encoded at genome coordinates 68, 39, and the putative main coding regions, which for pTP is an open reading frame (ORF) from map units (m.u.) 29.4 to 23.9 and for AdPol is an ORF between m.u. 22.9 and 14.2.

Because of the very small amounts of the E2B mRNAs and proteins synthesized during adenovirus infection, very little was known previously about the contributions of each of the exons to the resulting AdPol or pTP polypeptides. Linker-scanning mutations within the pTP ORF have suggested that a mutation upstream of the first AUG caused a temperature-sensitive pTP (8). In a recent paper that described the cloning of AdPol (30a), DNA sequences contained within the exon at genome coordinate 39 were shown to be essential for expression of active AdPol protein. To assess the importance of the upstream exons to the synthesis of pTP, plasmids containing the large pTP ORF and various DNA fragments 5′ to it were constructed and tested for expression of a pTP that would be functional for the initiation of viral DNA replication.

In this manuscript, the synthesis of the 80-kDa pTP from cloned DNA and its activity as a functional polypeptide in a cell-free replication system is described. This expressed pTP was shown to function in the initiation of AdDNA-pro replication, specifically at the origins at the ends of the viral DNA, in a reaction that also required AdPol. This initiation complex was able to function in the specific elongation of the AdDNA-pro template, in a reaction that also required the addition of adenovirus DBP. Functional pTP activity was dependent on viral sequences from genome coordinate 39 as well as the main ORF.

MATERIALS AND METHODS

Bacteria and plasmids. Cloned adenovirus type 2 (Ad2) DNA segments were inserted in a plasmid, p91023 (20, 36). The resulting constructs are shown in Fig. 1. Plasmid DNA was grown in *Escherichia coli* DH1 (16) and isolated by the alkaline lysis method (1) followed by banding two times in CsCl-ethidium bromide density equilibrium gradients.

Immune precipitation of pTP from extracts of transfected COS cells. At 43 h after transfection of COS cells with 10 μg of CsCl-purified plasmid DNA per 60-mm plate, cells were labeled for 45 min with [^{3}H]leucine (15, 17). The cells were harvested and prepared for immunoprecipitation with anti-

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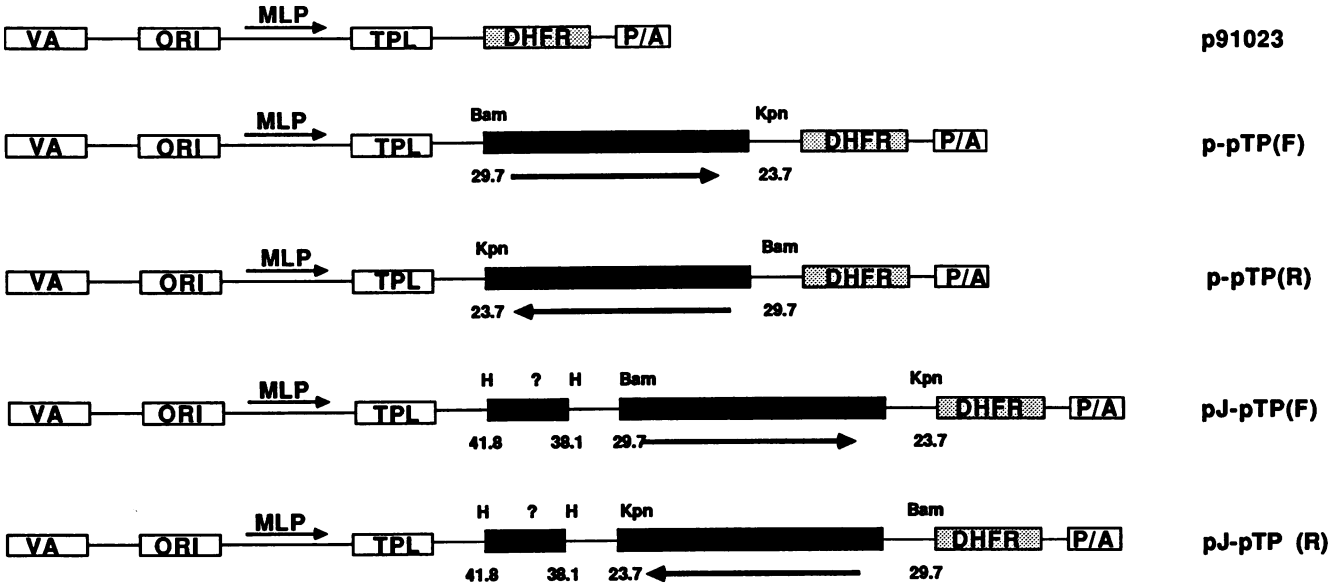

FIG. 1. Plasmids for transient expression of pTP. Adenovirus DNA segments, cloned into the p91023 vector and expressed by transfection of COS cells (13), are shown as black boxes, with restriction endonuclease cleavage sites and genome coordinates designated. Plasmid p-pTP (F) contained only the main ORF for pTP (a *Bam*HI-*Kpn*I fragment, between genome coordinates 29.7 and 23.7); plasmid p-pTP (R) was identical to p-pTP (F) except that the *Bam*HI-*Kpn*I fragment was reversed. Plasmid pJ-pTP (F) contained the *Hin*dIII J fragment, which encodes the putative exon at genome coordinate 39 upstream of the main ORF; plasmid pJ-pTP (R) was identical to pJ-pTP (F) except that the *Bam*HI-*Kpn*I fragment was reversed. The *Hin*dIII J fragment is marked ? because its contribution to the expression of functional pTP was being tested. Features of the vector important for transient expression include: the simian virus 40 (SV40) origin for replication and its enhancer sequences (ORI) to increase plasmid copy number and transcription, the Ad2 major late promoter (MLP), the Ad2 tripartite leader (TPL) with a donor splice site from leader segment 1 upstream of an acceptor splice site, the Ad2 VA_I and VA_{II} genes (VA) which enhance translation of mRNA containing the tripartite leader (21, 29), the dihydrofolate reductase gene (DHFR), and the simian virus 40 polyadenylation site (P/A).

body directed against the Ad2 55-kDa terminal protein (TP) (14) as described previously (30a).

Preparation of crude extracts of pTP and AdPol from transfected COS cells. COS cells, transfected with 20 μg of CsCl-purified plasmid DNA per 100-mm plate (15), were harvested 48 to 52 h after transfection by being washed with TBS and scraped off the plates. After the cells were collected by centrifugation, a crude cytoplasmic extract was prepared by Dounce homogenization (2). Nuclear extracts of pTP were 0.1 M NaCl extracts of nuclei isolated after the Dounce step. The plasmid used to transfect COS cells for preparation of crude cell extracts that contain AdPol activity (pJ_1-pol) has been described previously (30a).

Assay for initiation of adenovirus DNA replication. The initiation reaction for DNA synthesis was a modification of that described by Lichy et al. (23). The reaction contained Ad35DNA-pro (0.15 μg), uninfected HeLa nuclear extract, 10% glycerol, 20 mM NaCl, [α-^{32}P]dCTP (15 μCi, 400 Ci/mmol), 5 mM $MgCl_2$, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4), 3 mM rATP, 0.5 mM dithiothreitol, and 0.1 mM aphidicolin (Sigma Chemical Co.). Various preparations of AdPol and pTP (either from infected HeLa cells or from transfected COS cells) were added to the reaction mix in a final volume of 50 μl, as described in the legend to Fig. 3. After a 1-h incubation at 30°C, the reaction was terminated by the addition of an equal volume of 1% sodium dodecyl sulfate (SDS)–10 mM Tris (pH 8.0)–1 mM EDTA. After 5 min at 4°C, 400 μl of a solution containing 2% Triton X-100, 50 mM Tris (pH 8.1), 1 mM EDTA, 1 mg of ovalbumin per ml, and 3 μl of anti-TP antibody (14) was added, followed by incubation overnight at 4°C. The immune complexes were processed and washed prior to electrophoresis on 10% SDS-polyacrylamide gel electrophoresis (PAGE) as described previously (30a).

Assay for elongation of adenovirus DNA. The elongation of viral DNA in vitro was measured by using the end fragment, origin-specific assay described previously (18, 30a). In this assay, the Ad35DNA-pro terminal fragments, *Sma*I-B and -G (35), were preferentially labeled in a virus-specific in vitro reaction that initiated DNA synthesis at the origins at each end of the DNA. This reaction required AdPol, pTP, DBP, and host factors from uninfected HeLa cell nuclei. The COS cell extracts from cells transfected with an AdPol construct (pJ_1-pol) were previously shown to be active in this in vitro adenovirus DNA replication system (30a).

RESULTS

Transient expression of pTP in COS cells. Various adenovirus DNA fragments were cloned into p91023 downstream of the adenovirus major late promoter (Fig. 1). COS cells transfected with the plasmids described above were labeled with [^{3}H]leucine, and the pTP-related proteins were immunoprecipitated from the resulting lysates. When analyzed with anti-TP antibody (14), the pJ-pTP (F) and p-pTP (F) constructs contained equivalent amounts of immunoprecipitable proteins (Fig. 2). When the coding region for the pTP ORF was inserted in the antisense orientation, no pTP-related protein was observed.

Initiation of viral DNA synthesis on pTP translated from transfected plasmids. Figure 3 shows the nature of the pTP requirement to form the 80-kDa specific initiation complex for adenovirus DNA replication in vitro. Only extracts from COS cells transfected with pJ-pTP (F) were able to cova-

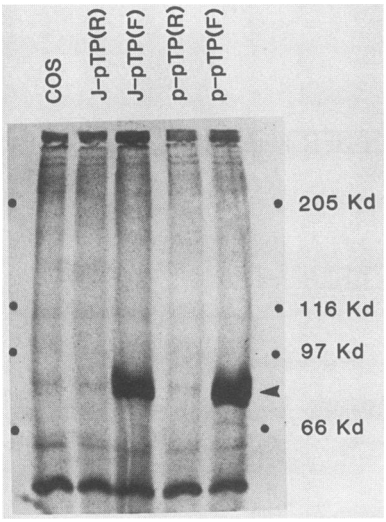

FIG. 2. Immunoprecipitation of pTP from extracts of transfected COS cells. Crude cell lysates of transfected COS cells labeled with [^{3}H]-leucine at 43 h posttransfection were prepared and immunoprecipitated as described previously (30a). The washed immunoprecipitates were analyzed by SDS-PAGE. The arrow shows the position of apparently full-length material. The positions of the size markers (in kilodaltons) are indicated by the dots on both sides. The autoradiogram was exposed for 8 days.

lently attach [α-^{32}P]dCTP. The reaction required the pTP product of virus sequences from both 39 m.u. (*Hin*dIII J fragment) and the pTP ORF (m.u. 29.7 to 23.7) in the correct orientation. There were no significant differences in the activities of nuclear or cytoplasmic extracts. The active pTP extracts (Fig. 3, lanes 1 and 3) contained small amounts of a 55-kDa protein in addition to the 80-kDa product. The 55-kDa protein was not detected in reactions with the pTP-AdPol complex isolated from adenovirus-infected HeLa cells (Fig. 3, lane 9).

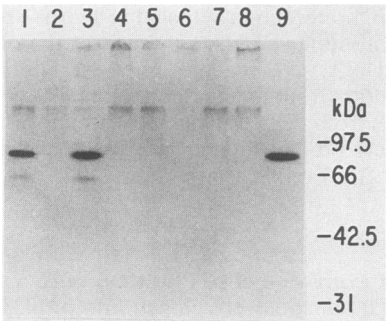

FIG. 3. Initiation of adenovirus-specific DNA replication with pTP produced from cloned plasmid constructs. The initiation reaction was performed with crude extracts of pTP, isolated as described in Materials and Methods. The amounts given for each construct refer to the total protein concentration of the crude extract added to the reaction; n and c indicate nuclear and cytoplasmic extracts, respectively. All lanes except 6 and 9 contained crude extracts of AdPol (6.9 μg) synthesized in transfected COS cells. Other additions were: lane 1, 5.6 μg of pJ-pTP (F) (n); lane 2, 2.8 μg of pJ-pTP (R) (n); lane 3, 1 μg of pJ-pTP (F) (c); lane 4, 10 μg of pJ-pTP (R) (c); lane 5, no additions; lane 6, 5.6 μg of pJ-pTP (F); lane 7, 5.4 μg of p-pTP (F) (n); lane 8, 6.2 μg of p-pTP (F) (c); lane 9, the pTP-AdPol complex purified from Ad2-infected HeLa cells. After incubation for 1 h at 37°C, the reactions were terminated and the samples were processed as described in Materials and Methods.

Elongation of AdDNA-pro with pTP translated from various transfected plasmids. The functional activity of crude lysates of COS cells transfected with several different pTP plasmid constructs is shown in an in vitro adenovirus DNA replication assay (Fig. 4). The assay measured the incorporation of [α-^{32}P]dTTP into the end fragments of *Sma*I-cleaved Ad35DNA-pro; each of the end fragments (*Sma*I-B and -G) contains an origin for viral DNA replication and was preferentially labeled when active pTP and AdPol were added to the standard assay reaction mix. The data demonstrate that active pTP required sequences in the m.u. 39 region in addition to the pTP ORF to produce functional protein. Reversing the orientation of the ORF completely eliminated pTP synthesis and activity (Fig. 4A). Furthermore, the pTP activity obtained from the cloned DNA segments resulted in covalent attachment of protein (presumably pTP) to newly replicated labeled viral DNA. Since the DNA fragments failed to enter the gel without pronase treatment, the disappearance of the specific radioactive bands (*Sma*I-B and -G) (Fig. 4B, lane 1) indicates that the progeny DNA was covalently linked to protein. An identical reaction mix (lane 3) treated with pronase prior to PAGE showed both labeled product bands (*Sma*I-B and -G), indicating faithful and specific viral DNA replication.

DISCUSSION

In this manuscript, the contribution of the exon at genome coordinate 39 to the production of functional pTP from cloned DNA in plasmid vectors has been determined. Although the addition of this upstream exon had no apparent effect on the molecular mass or the amounts of an 80-kDa polypeptide that could be immunoprecipitated with antibody against TP (14), there was a dramatic effect on the activity of the expressed pTP: only the construct containing the *Hin*dIII J fragment (genome coordinate 39) was active. Thus, the

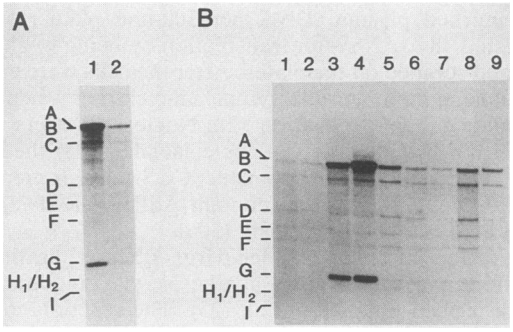

FIG. 4. Adenovirus-specific DNA replication with Ad2 pTP synthesized from cloned plasmid constructs. The assay for specific synthesis of viral DNA on the terminal fragments of Ad35DNA-pro was performed as described in Materials and Methods. The amounts given for each extract refer to the total protein concentration of the crude extract added to the reaction; n and c indicate nuclear and cytoplasmic extracts, respectively. Each reaction contained Ad2 DBP isolated from infected cells and crude extracts of AdPol (4.2 μg) prepared by transfection of COS cells. The reactions in all lanes were terminated with SDS and pronase, except for lanes B1 and B2, from which the pronase was omitted. Letters on the left-hand side of the panels refer to the *Sma*I-digested fragments of Ad35DNA-pro. (A) Lanes: 1, 5.6 μg of pJ-pTP (F) (n); 2, 5.6 μg of pJ-pTP (R) (n). (B) Lanes: 1, 5.2 μg of pJ-pTP (F) (n); 2, no pTP added; 3, 5.2 μg of pJ-pTP (F) (n); 4, 1.0 μg of pJ-pTP (F) (c); 5, no pTP; 6, 5.8 μg of p-pTP (R) (n); 7, 6.6 μg of p-pTP (R) (c); 8, 5.4 μg of p-pTP (F) (n); 9, 6.2 μg of p-pTP (F) (c).

contribution of amino acids from both the *Hin*dIII J fragment and upstream of the first AUG in the pTP ORF must be small enough not to affect the mobility of the resulting functional pTP in SDS-PAGE.

In the initiation reaction, covalent attachment of [α-^{32}P]dCMP to the 80-kDa pTP was demonstrated. In addition, there was a small amount of ^{32}P in a 55-kDa protein (Fig. 3, lanes 1 and 3). The 55-kDa polypeptide presumably represents TP, which, like its precursor pTP molecule, contains the serine at amino acid 580 that can bind dCMP covalently (6). However, this dCMP-binding domain is also contained in the truncated p-pTP (F) polypeptide lacking the amino end of the pTP produced from pJ-pTP (F). Since the p-pTP (F) contains this dCMP-binding domain but is functionally inactive in the 80-kDa–dCMP reaction, we presume that the labeled 55-kDa protein in Fig. 3 (lanes 1 and 3) resulted from proteolysis of the functional 80-kDa polypeptide after [^{32}P]dCMP labeling rather than direct dCMP binding and labeling of the 55-kDa protein. In adenovirus-infected cells, the pTP to TP conversion appears to be mediated by a viral protease which should be totally absent from the transfected COS cell extracts. These results suggest that a protease from COS cells can also convert small amounts of pTP to TP.

The probable organization of the gene-coding capacity of early region 2B is shown in Fig. 5. Together with previous data obtained by analysis of transient expression of AdPol (30a), the initial AUG codon for both pTP and AdPol is most likely encoded within the exon at genome coordinate 39. Following RNA splicing of this exon to the beginning of the ORFs for pTP and AdPol, the amino acids encoded at m.u. 39 would be attached to the pTP and the AdPol polypeptides encoded within their respective ORFs. Since there is no apparent difference in the molecular weight of pJ-pTP and p-pTP, the distance between the AUG within the upstream exon and the first AUG within the pTP ORF must be relatively short.

In an earlier study of linker insertion mutants of pTP, Freimuth and Ginsberg (8) studied two pTP mutants that were defective for adenovirus DNA replication and were mapped to sequences upstream of the first AUG in the pTP ORF. The results reported here, that upstream sequences encoded at genome coordinate 39 are important for expression of pTP, are consistent with their data. The contribution of exons upstream of 39 m.u. was not tested for the pTP constructs. However, in a previous manuscript (30a), the exon at 68 m.u. was tested for its effect on AdPol expression but did not contribute to either the size or the activity of AdPol.

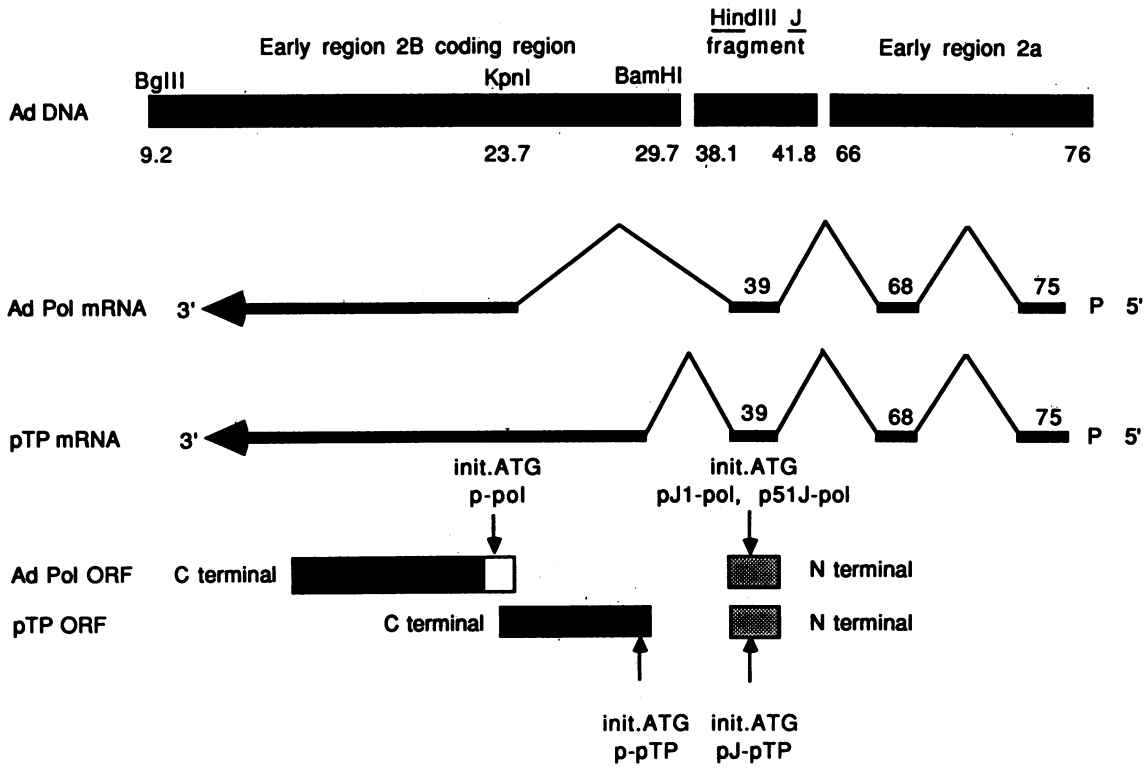

FIG. 5. Structure and predicted coding regions of the mRNA from which functional pTP and AdPol are synthesized. Adenovirus (Ad) DNA segments from which Ad2 pTP and AdPol exons are derived are shown as solid rectangles on the top line; the approximate genome coordinate of each exon is shown below the segment. Below the representation of these DNA molecules, the structures of the processed mRNAs that encode pTP or AdPol are shown as thick arrows interrupted by introns (thin lines) and pointing from 5' to 3'. These structures contain all of the exons mapped by Stillman et al. (32). The promoter for transcription of early region 2 is designated P. The highly conserved regions of the pTP and AdPol gene sequences are shown as solid rectangles underneath the arrow. The AdPol ORF remains open further upstream of the highly conserved region in Ad2 (12), Ad5 (5), Ad7 (7), and Ad12 (30). This less conserved upstream region in AdPol is essential for AdPol enzymatic activity (30a) and is shown as an open rectangle. At genome coordinate 39, the stippled box is shown at the position of a hypothetical ORF within the exon that would supply the initiator AUG to both pTP and AdPol constructs containing the *Hin*dIII J fragment in the correct orientation. The probable sites of the first AUG used for expression by the various constructs in Fig. 1 are shown with vertical arrows and are compared with analogous sites in the AdPol constructs studied previously (Shu et al., in press).

Since enzymatically active pTP and AdPol can be produced from cloned DNA, additional aspects of adenovirus DNA replication can now be explored. Specific mutations can be introduced into the pTP and AdPol proteins to identify and map functional and structural domains important to both adenovirus-specific and generalized DNA replication activities.

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