*Proc. Natl. Acad. Sci. USA*
Vol. 86, pp. 6116–6120, August 1989
Biochemistry

# Dissection of functional domains of adenovirus DNA polymerase by linker-insertion mutagenesis

(DNA replication)

M. CHEN* AND M. S. HORWITZ*†‡

Departments of *Cell Biology, †Microbiology-Immunology, and ‡Pediatrics, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461

*Communicated by Frank Lilly, May 25, 1989*

**ABSTRACT** **Linker-insertion mutations were introduced into the cloned adenovirus DNA polymerase gene and the functional effects on the initiation and elongation of DNA *in vitro* were measured. Essential regions of the polymerase appear to be scattered in patches across the entire molecule and are not limited to the five regions of homology shared with a variety of other replicating polymerases. Thus, the adenovirus DNA polymerase presumably contains active sites that must be formed by distant interactions across the polymerase molecule.**

Adenovirus (Ad) type 2 contains a linear, duplex, 36-kilobase-pair (kb) DNA with inverted terminal repeats and a 55-kDa terminal protein (TP) covalently bound to each 5′ end (1, 2). Initiation of DNA replication occurs by a protein-priming mechanism in which the first deoxynucleotide, dCMP, is covalently attached to an 80-kDa precursor of the TP (pTP) (2, 3). The replication of Ad DNA *in vitro* requires six proteins of which three, the 80-kDa pTP, the 140-kDa Ad DNA polymerase (Ad Pol), and the 59-kDa Ad DNA-binding protein (DBP), are virus-encoded (4–7). Three host proteins, nuclear factors (NF) I–III, have been purified from uninfected HeLa cells. NFI and NFIII specifically recognize the Ad origin of replication at each end of the template and stimulate replication (8–10). NFII is a type I DNA topoisomerase (11).

Ad Pol is an essential enzyme for both the initiation and elongation steps of DNA replication; it has been characterized biochemically and genetically by using the Ad5 temperature-sensitive, DNA-negative mutants ts149 and ts36 (7, 12, 13). Ad Pol is isolated as an equimolar complex with pTP from Ad-infected cytoplasmic extracts but can be separated from pTP by glycerol gradient sedimentation in the presence of 1.7 M urea (14). Ad Pol is resistant to aphidicolin, a specific inhibitor of the eukaryotic DNA polymerases $\alpha$ and $\delta$, and is a multifunctional enzyme for which the following activities have been characterized: (*i*) an aphidicolin-resistant DNA polymerase reaction that adds deoxynucleosides to 3′ ends of gapped DNA (14, 15); (*ii*) a *de novo* chain-initiation activity measured as covalent complex formation between the first deoxynucleotide and pTP (pTP-dCMP) (3, 16); (*iii*) an Ad DBP-dependent elongation reaction on poly(dT)·oligo(dA) template primers (17, 18); (*iv*) a DNA chain-elongation activity on double-stranded Ad DNA (19); and (*v*) a 3′→5′ exonuclease activity (18). In addition, Ad Pol binds to pTP and has been postulated to have a binding site for DNA, deoxynucleoside triphosphates (dNTPs), pyrophosphate, and TP during initiation. Ad Pol may also have a binding site for the Ad DBP.

Comparison of the deduced primary structure of the replicating polymerases Ad Pol, human DNA polymerase $\alpha$, and the viral DNA polymerases from herpes simplex and vaccinia as well as bacteriophages T4 and $\phi$29 has identified as many as six regions that are highly conserved and in the same linear array (20, 21). These observations have led to speculation that homologous regions represent important functional domains of the polymerase polypeptides and that they may be derived from a common origin. Despite the importance of Ad Pol for DNA synthesis *in vivo* and *in vitro*, only small amounts of the enzyme can be purified from large quantities of infected tissue culture cells. This has hampered detailed studies of the structural aspects and functional domains of the protein. Very little is known about the interaction of Ad Pol with its various substrates, its DNA template, or other proteins involved in the DNA replication complex. Information of this nature would not only be of fundamental interest for Ad replication but also be useful in understanding the mechanism of action of other DNA polymerases. Ad Pol has been successfully cloned, expressed in COS-1 cells from an expression vector (p91023), and shown to specify an enzymatically active DNA polymerase in a cell-free replication system (22). With the production of an active Ad Pol *in vitro*, it has become possible to systematically explore the functional domains of this polypeptide and investigate the significance of evolutionarily conserved amino acid sequences by rapid and selective *in vitro* mutagenesis of the cloned gene.

We have constructed a series of 16 linker-insertion mutations within the coding region of the Ad Pol gene and determined the effect of each mutation in three independent *in vitro* reactions. The results indicate that essential regions for the individual Ad Pol enzymatic reactions map in patches across its entire coding region. Therefore, the linear two-domain models that exist for *Escherichia coli* DNA polymerase I or the murine reverse transcriptase probably do not apply to Ad Pol (23–25).

## MATERIALS AND METHODS

**Cells and Plasmids.** The plasmid $pJ_1$-pol, which contains the coding region for Ad2 Pol, has been described (22). All plasmid DNAs were grown in *E. coli* DH1 and isolated by alkaline lysis (26) followed by banding in CsCl/ethidium bromide equilibrium density gradients. The CMT4 cell line, originally isolated by Gerard and Gluzman (27), contains the gene for the simian virus 40 (SV40) large tumor (T) antigen behind a metallothionein promoter.

**Construction of Linker-Insertion Mutations in the Ad Pol Gene.** The strategy used to construct a series of in-phase linker-insertion mutations was based on the method of Stone *et al.* (28). A 20-$\mu$g sample of $pJ_1$-pol DNA was partially digested with *Hin*cII, *Sca* I, *Rsa* I or *Alu* I restriction enzyme in the presence of 0.8–1.2 $\mu$g of ethidium bromide per $\mu$g of DNA. The amount of ethidium bromide was titrated to limit

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: Ad, adenovirus; Ad Pol, Ad DNA polymerase; TP, terminal protein; pTP, precursor to TP; Ad35 DNA-pro, Ad type 35 DNA covalently linked to TP at each 5′ end; DBP, DNA-binding protein; NF, nuclear factor; wt, wild type.

DNA digestion to circularly permuted, full-length, linearized DNA molecules. The linearized molecules were separated from nicked circles by preparative gel electrophoresis in low-melting-temperature agarose, and the DNA band corresponding to full-length linear DNA (14.5 kb) was identified by comigration with $pJ_1$-pol DNA linearized at a single *Eco*RI site. Nonphosphorylated *Bam*HI linkers [d(5'-CCGCGGATTCCCGCG-3')] were ligated onto the linearized DNA by linker tailing (29). Excess linkers were removed by precipitation with spermine (30) and the linker-tailed DNAs were rehybridized. A sample of this DNA was used to transform competent *E. coli* DH1 and the tetracycline-resistant bacterial colonies were screened for the presence of plasmids containing a new *Bam*HI site. Digestion of $pJ_1$-pol DNA with *Bgl* II and *Bam*HI produced fragments of 5.5, 4, and 3 kb and a 1-kb doublet. Insertion of the linker within the main coding region of the Ad Pol gene (*Bgl* II fragment) resulted in the cleavage of the 5.5-kb fragment and the sizes of the resulting fragments indicated the site of the inserted linker. For some linker-insertion mutants, digestion with *Bam*HI alone was used to map the insertion site in addition to the *Bgl* II/*Bam*HI double digestion. Comparison of the sizes of the generated fragments with those predicted from the published DNA sequence of Ad2 Pol allowed us to identify the restriction sites containing the inserted *Bam*HI linker (31).

**Preparation of Crude Extracts of Each Ad Pol Linker-Insertion Mutant from Transfected CMT4 Cells.** Transfection experiments using 60 μg of purified plasmid DNA in each 150-mm dish of CMT4 cells were performed essentially as described (32). Five hours after the addition of DNA, the transfected cells were washed with phosphate-buffered saline (PBS) and incubated with medium containing 1 μM $CdSO_4$ and 100 μM $ZnCl_2$. Forty-eight to 52 hr after transfection, the cells were washed with PBS, scraped from the plates, and collected by centrifugation. A crude cytoplasmic extract was prepared by Dounce homogenization (4).

**Immunoblot Analysis.** Crude cytoplasmic extracts from CMT4 cells were resolved by SDS/7.5% PAGE, transferred to a nitrocellulose filter, and probed with antibody to Ad Pol (33), which was detected with alkaline phosphatase-conjugated anti-rabbit IgG as the second antibody (see Fig. 6). The amounts of the Ad Pol proteins were quantitated by scanning densitometry of the immunoblots.

**Synthesis of Poly(dA) by Ad Pol.** The DBP-dependent synthesis of poly(dA) by Ad Pol on poly(dT) templates has been described (18). Reaction mixtures (50 μl) contained 50 mM Tris/HCl buffer (pH 7.5), 4 mM dithiothreitol, 1 μg of bovine serum albumin, 9 mM $MgCl_2$, 5 mM ATP, 8 μM [$\alpha$-$^{32}$P]dATP (2000 cpm/pmol), 0.33 μg of poly(dT), and 0.33 μg of oligo(dA). Reaction mixtures were prepared by annealing primer and template for 10 min at 65°C, followed by the addition of the other components and incubation for 15 min at 37°C. The DNA synthesis reaction was started by the addition of DBP (0.2 μg) and Ad Pol-containing cytoplasmic extract. The mixtures were incubated at 37°C for 60 min and acid-insoluble radioactivity was measured.

**Initiation of Ad DNA Replication.** The initiation reaction for DNA synthesis was performed essentially as described (3, 22). Reaction mixtures (50 μl) contained various extracts of Ad Pol mutants, Ad type 35 DNA covalently linked to TP at each 5' end (Ad35 DNA-pro, 0.15 μg), the DEAE-cellulose fraction of nuclear extract from uninfected HeLa cells, wild-type (wt) pTP extract from transfected CMT4 cells (J-pTP), 10% (vol/vol) glycerol, 20 mM NaCl, 15 μCi of [$\alpha$-$^{32}$P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq), 5 mM $MgCl_2$, 25 mM Hepes (pH 7.4), 3 mM ATP, 0.5 mM dithiothreitol, and 0.1 mM aphidicolin. After a 1-hr incubation at 30°C, the reaction was terminated by the addition of 50 μl of 1% SDS/10 mM Tris·HCl, pH 8/1 mM EDTA. After 5 min at 4°C, 400 μl of 10 mM Tris·HCl, pH 8/1 mM EDTA/0.1% ovalbumin and 3 μl of anti-TP antibody were added and the mixture was incubated overnight at 4°C. The immunocomplexes were precipitated with formalin-fixed *Staphylococcus aureus*, processed, and washed prior to SDS/10% PAGE.

**Ad-Specific DNA Replication.** The end-fragment, origin-specific assay for Ad DNA replication *in vitro* has been described (19). Ad35 DNA-pro (0.15 μg) was first cleaved with restriction endonuclease *Sma* I (5 units) for 2 hr at 30°C before the replication reaction was initiated by the addition of cytoplasmic extracts of the various Ad Pol mutants; the DEAE-cellulose fraction of nuclear extract from uninfected HeLa cells; 0.8 μg of Ad2 DBP; J-pTP cytoplasmic extract from transfected CMT4 cells; 3 mM ATP; 5 mM $MgCl_2$; 50 μM dATP, dGTP, and dCTP; 62.5 pmol of [$\alpha$-$^{32}$P]dTTP (1.25 μCi); and 25 mM Hepes (pH 7.5) in a total volume of 50 μl. The mixture was incubated at 37°C for 60 min, and the reaction was stopped by incubation with 0.2% SDS and 100 μg of Pronase per ml for 45 min at 37°C. After precipitation of the DNA in ethanol and subsequent solubilization in 25 μl of 80 mM Tris/80 mM phosphate/8 mM EDTA, pH 8 (TPE buffer), the DNA was analyzed by 0.7% agarose gel electrophoresis in TPE buffer, followed by autoradiography. In this assay, the Ad35 DNA-pro terminal fragments are *Sma* I B and G (34), which are preferentially labeled because each contains an origin for DNA replication. The amount of specific synthesis was quantitated by scanning the autoradiograms and measuring incorporation into the G band corrected for the nonspecific incorporation into the F band.

## RESULTS

**Construction of Ad Pol Linker-Insertion Mutations.** Mutagenesis was conducted on the $pJ_1$-pol plasmid, which contains the p91023 expression vector, the main coding region of Ad Pol in a long open reading frame, and the short upstream region at 39 map units that encodes the N-terminal 3 amino acids in a *Hin*dIII DNA fragment. Structural features of this Ad Pol gene and the sites of linker insertions are shown in Fig. 1. The enzymes *Alu* I, *Rsa* I, *Sca* I, and *Hin*cII were used independently to cut $pJ_1$-pol to ensure a good distribution of linkers inserted throughout the Ad Pol gene. Sixteen of the linkers were within the Ad Pol gene, but one, 37A, was positioned after the termination codon. We were able to isolate all of the potential insertions at *Hin*cII and *Sca* I sites, but we isolated insertions at only 5 of 8 *Rsa* I sites in 110 clones screened and 15 of 34 *Alu* I sites in 250 clones screened. The use of the 12-nucleotide *Bam*HI linker does not specify a stop codon in any of the three reading frames. The nucleotide sequence of the Ad2 Pol gene was used to predict the amino acid sequence of each mutant protein that resulted from linker insertion (31). At 12 restriction sites, insertion occurred between two amino acid codons, whereas at four sites insertion was within a codon (Fig. 1).

**Effect of Ad Pol Linker-Insertion Mutations on Poly(dA) Synthesis.** With poly(dT) as template and oligo(dA) as primer, DNA synthesis by Ad Pol is stimulated 10- to 100-fold by the 59-kDa Ad DBP (18). This enhancement is specific for the cognate Ad DBP on this template and is a much more specific assay for Ad Pol than the reaction on activated DNA in the presence of aphidicolin. The poly(dT)·oligo(dA) assay probably involves the specific interaction between Ad Pol and Ad DBP but does not require pTP or any of the nuclear factors from uninfected cells. The activity of Ad Pol linker-insertion mutants on poly(dA) DNA synthesis is shown in Fig. 2. Four mutants (6R, 69A, 9A, and 14S) were fully active; two of the mutations (6R and 69A) are upstream of the first methionine codon in the long open reading frame (see ATG, 8358 in Fig. 1), in a region that by deletion mutagenesis was shown to be essential for Ad Pol function (22, 35). The activity of eight

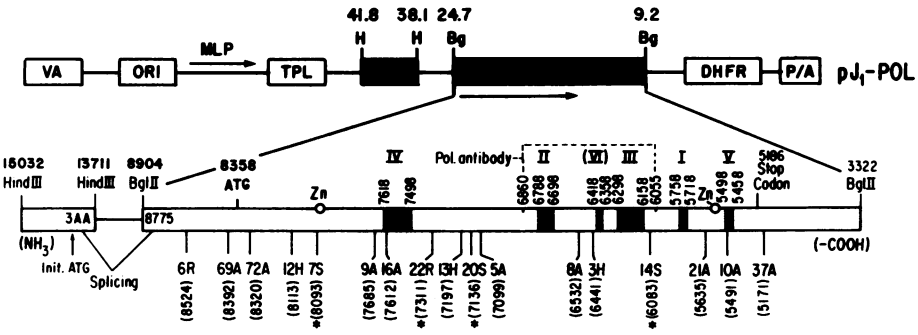


FIG. 1. (*Upper*) A schematic of $pJ_1$-pol, the p91023-derived expression plasmid containing the two Ad Pol inserts (22). (*Lower*) Sites within the Ad Pol gene that were mutagenized by insertion of a dodecameric *Bam*HI linker. Numbers in parentheses indicate positions of restriction sites. Letter R, H, S, or A appended to the number of each mutant represents *Rsa* I, *Hin*cII, *Sca* I, or *Alu* I restriction endonuclease sites, respectively. Regions of homology between Ad Pol and a variety of other DNA polymerases are shown as solid rectangles, designated by roman numerals. Two consensus sequences for potential metal-binding sites are indicated (Zn), and the Ad Pol domain to which we had made antibody used for immunoblot analysis is bracketed. Mutations designated by asterisks represent a Tyr→Ser change and insertion of Arg-Ile-Arg-Asp in the mutant protein; mutations at all other sites resulted in insertion of Arg-Gly-Ser-Ala without a change in the existing sequence. The initiating ATG is shown in the upstream *Hin*dIII fragment and the first ATG in the long open reading frame is shown between mutations 69A and 72A.

mutants (72A, 12H, 16A, 13H, 8A, 3H, 21A, and 10A) was <15% of wt activity; two of the mutations (16A and 10A) are located in conserved regions IV and V, respectively, which share homology with a variety of other DNA polymerases (20, 21). The other four mutants (7S, 22R, 20S, and 5A) retained 35–70% of wt Ad Pol activity. Mutant 7S, which retained 70% of the wt activity, mapped within one of the two putative DNA-binding domains of Ad Pol. In the 7S mutant, the amino acid sequence motif $CX_2C$ is changed to $CX_6C$.

**Effect of Ad Pol Mutations on Initiation of Ad DNA Replication.** The initiation reaction, which is determined by the covalent addition of [$\alpha$-$^{32}$P]dCMP to the 80-kDa pTP as detected by SDS/PAGE, requires Ad DNA-pro as template, Ad Pol, and pTP as well as the NFI and -III from uninfected cells. This assay does not require Ad DBP. The effect of various Ad Pol linker-insertion mutations on the formation of the 80-kDa initiation complex is shown in Fig. 3. When corrected for the amount of Ad Pol in each reaction mixture, four mutants (6R, 69A, 9A, and 22R) were approximately as active as wt; three mutants (5A, 20S, and 14S) had partial activity. The rest of the mutants were essentially inactive; among them, two (16A and 10A) have mutations in conserved regions IV and V, respectively; one mutation (7S) is in the potential metal-binding domain described above. In addition to the 80-kDa product, 62-kDa and 55-kDa proteins were produced, presumably by proteolysis of the 80-kDa polypeptide. This proteolysis may be similar to the conversion of the 80-kDa pTP to the 55-kDa TP, which is mediated by a virally coded protease in permissively infected cells (36).

**Effect of Ad Pol Mutations on Ad-Specific DNA Elongation.** Specific DNA replication on Ad DNA-pro, which maintains the fidelity of intracellular Ad DNA replication (5, 6), requires the three viral gene products (Ad Pol, pTP, and DBP) and at least three factors purified from uninfected nuclear extracts. The assay measures the incorporation of [$\alpha$-$^{32}$P]dTMP into the end fragments (*Sma* I fragments B and G) of *Sma* I-digested Ad35 DNA-pro, (Fig. 4, lane 1). Any nonspecific radioactive incorporation, such as repair reactions on the internal DNA fragments, can be distinguished by the labeling of all of the restriction fragments with an amount of radioactivity that corresponds directly to their molecular sizes (lanes 11 and 12). The functional activity of various Ad Pol linker-insertion mutants on specific Ad DNA replication is shown in Fig. 4. Because the B fragment can be labeled with a significant amount of radioactivity, either by specific incorporation or, because of its large size, by nonspecific incorporation, only the G fragment was used for quantitation of elongation activity (Fig. 5). Two linker-insertion mutants (6R and 69A) retained 85–100% of wt activity; three others (9A, 22R and 14S) were 15–65% as active as wt. The rest of the insertion mutations completely abolished the Ad Pol activity in specific DNA replication. The results of the three functional assays are summarized in Fig. 5.

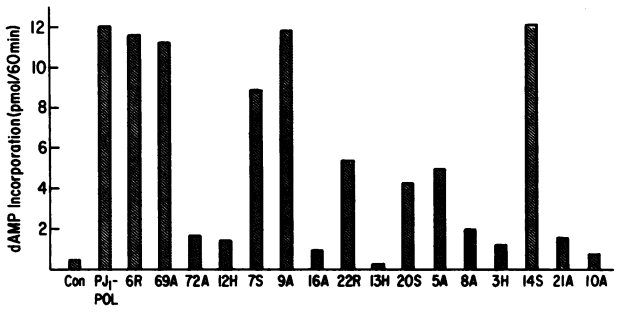


FIG. 2. Poly(dA) synthesis by Ad Pol linker-insertion mutants. The designation of mutants is shown in Fig. 1. All reaction mixtures contained Ad2 DBP (0.2 $\mu$g) isolated from infected HeLa cells, as well as 3 $\mu$l of cytoplasmic extract from CMT4 cells transfected with the wt ($J_1$-pol) or a mutant Ad Pol expression plasmid. Con, negative control, the p91023 plasmid lacking the Ad Pol gene is shown in the first lane. All other results are normalized to the amount of Ad Pol in the wt extract.

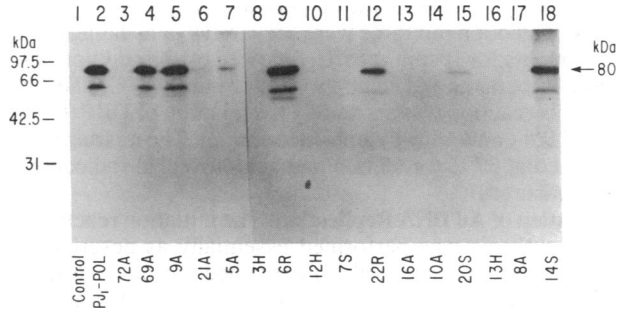


FIG. 3. Initiation of Ad-specific DNA replication with Ad Pol linker-insertion mutants. The initiation reaction was performed with equal volumes of Ad Pol-transfected CMT4 cytoplasmic extracts. In addition, all reaction mixtures contained an equal amount of cytoplasmic extract of wt pTP from transfected cells and a nuclear extract from uninfected HeLa cells. Control, cytoplasmic extract of CMT4 cells transfected with the p91023 plasmid without the Ad Pol insert; $pJ_1$-pol, plasmid containing the wt Ad Pol gene. After incubation for 1 hr at 37°C, the reactions were terminated and the samples were processed.

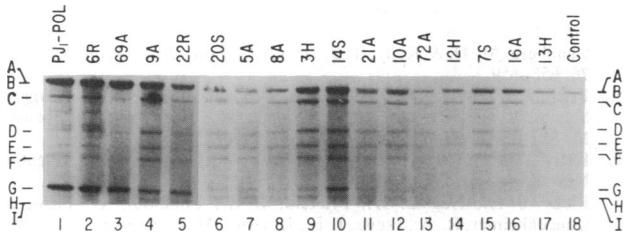


FIG. 4. Ad-specific DNA elongation with Ad Pol mutants. The assay for specific synthesis of viral DNA on the terminal fragments of Ad35 DNA-pro (B and G), each of which contains an origin for replication, was performed. Each reaction mixture contained Ad2 DBP purified from infected HeLa cells, pTP from transfected CMT4 cells, nuclear extract of uninfected HeLa cells, and an equal amount of Ad Pol protein (based on immunoblot analysis of an extract prepared from transfected cells). $pJ_1$-Pol, wt; control, p91023 plasmid without Ad Pol insert.

## DISCUSSION

Linker-insertion mutagenesis has proven to be a useful way to begin the examination of functional regions within Ad Pol. This approach has been successfully used to study functional domains within v-*fms* and v-*erbB* oncogene products as well as the human glucocorticoid receptor (37–39). The procedure is applicable to any cloned sequence and in the present study yielded a sizable collection of mutations with predetermined structural properties. Depending on the reading frame and surrounding nucleotides, different amino acids were inserted at the various sites (Fig. 1). All the insertion mutations reported here introduced similar changes in the hydrophobicity of the amino acids. Therefore, the different functional phenotypes are due most likely to the site rather than to the nature of insertion.

The results of any form of mutagenesis must be interpreted with some caution in the absence of specific knowledge of the effect of primary structure changes on the overall conformation of the polypeptide. However, most examples of site-specific mutagenesis recently reported suggest that the global conformation of the polypeptide is not affected. Even when increased susceptibility to proteolysis suggested a general structural change of the Ad Pol, we have found that the specific activity of some of these mutants measured in the enzymatic reactions described above have not been concomitantly decreased (data not shown). Such results can be obtained with considerable accuracy in the *in vitro* system, in which quantities of proteins and their enzymatic activity can be measured in reactions that maintain the fidelity of intracellular viral DNA replication. Some of these mutants would be difficult or impossible to propagate in virus for assay. However, it is premature to conclude that regions of Ad Pol that are sensitive to mutational inactivation are directly responsible for essential Ad Pol functions.

The 16 linker-insertion Ad Pol mutants can be separated into groups on the basis of activities, ranging from mutants that were unaffected in any of these reactions to those that were completely inactive in all assays. Two N-terminal mutations, 6R and 69A, are located upstream of the first methionine (ATG, 8358 in Fig. 1) of the main open reading frame, in a region whose deletion resulted in elimination of all Ad Pol activity (22, 35). However, neither of these two insertion mutations had any adverse effect on Ad Pol activity in any of the assays, presumably because they are in portions of the N-terminal region that are not required for any of the Ad Pol functions. Eight mutants mapping throughout Ad Pol were inactive in all assays. This may be due primarily to an alteration in an active site necessary for all the reactions or to effects on the tertiary structure of the macromolecule. Two of these eight mutations are located in the conserved regions IV and V, shared with other DNA polymerases, confirming the importance of some homology regions for DNA polymerase function. Previous results have shown that a 4-amino acid change in region I also inactivates all Ad Pol function (I. Joung, S. Hsu, J. Engler, and M.S.H., unpublished data). The 16A mutant (region IV) at base 7612 maps near the site previously identified for the Ad5 ts36 mutant (base 7623), which is defective for DNA replication at the nonpermissive temperature (40, 41). However, we have not been able to detect a temperature-sensitive phenotype by analyzing the activity of 16A at lowered temperature.

Five insertion mutants had dissociated activities in the various assays. The enzymatic reaction requiring the interaction of the most proteins, elongation activity on the end fragments of Ad DNA-pro, was inhibited most frequently, followed by decreased activity either in the initiation reaction or in the poly(dT)·oligo(dA) elongation assay. However, in most cases of partial or full activity in the poly(dT)·oligo(dA) assay, the mutants also retained some activity in the initiation reaction. The result can be explained by the difference in the complexity and requirements of each reaction. The end-fragment elongation reaction requires the integrity and activity of Ad Pol in multiple protein and DNA interactions, whereas for poly(dA) synthesis, Ad Pol must interact only with Ad DBP or the homopolymer. Therefore, changes in Ad Pol structure might alter its interaction with some proteins or the DNA-pro template but maintain other sites required for function on the homopolymer. Mutants in this group could aid in the identification of the domain that is needed for one reaction but not for another; however, additional mutants will be necessary to fully explore this issue.

In Ad Pol there are two consensus sequences that could fold into a "fingered" structure centered on a zinc ion (42, 43). The first sequence, at 8083, is CQYCARFYKSQHEC-SARRRDFYFHHINSH, which could fold into a loop structure coordinated by $Zn^{2+}$. This sequence contains the essential cysteines and histidines that are conserved in the four Ad

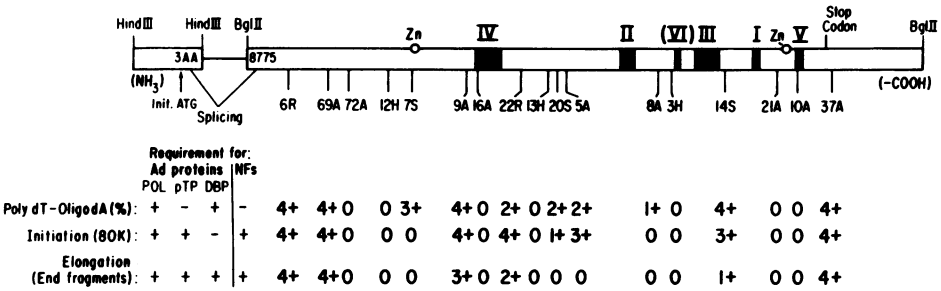


FIG. 5. Summary of structure–function studies of Ad Pol linker-insertion mutants. Ad-specific and host proteins required for each of the three reactions are shown together with the results of each assay. All quantities are normalized for the amount of the 140-kDa Ad Pol protein, determined in each extract by immunoblots with specific Ad Pol antibody (Fig. 6), and are expressed as quartiles. Activities: 4+, 75–100% of wt; 3+, 50–74%; 2+, 25–49%; 1+, 10–24%; 0, ≤10%.

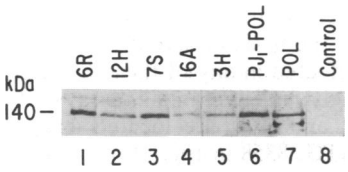


FIG. 6. Immunoblot analysis of Ad Pol linker-insertion mutants. Equal volumes of wt and various mutant Ad Pol cytoplasmic extracts from transfected CMT4 cells were analyzed by using antiserum to a LacZ–Ad Pol–chloramphenicol acetyltransferase fusion protein. Control, cytoplasmic extract of CMT4 cells transfected with p91023 plasmid without Ad Pol insert; $pJ_1$-pol, plasmid containing wt Ad Pol; POL, purified Ad2 Pol isolated from infected HeLa cells. The 140-kDa polypeptide is indicated and was quantitated in each lane by densitometry.

Pol serotypes already sequenced. The second sequence, which is encoded beginning at base 5615, is $CX_2CX_{23}CX_3C$. The 7S (8093) mutation carries an amino acid insertion in the first sequence and changes $CX_2C$ to $CX_6C$, destroying the putative metal binding site. This mutant, which is totally inactive in the end-fragment and initiation reactions but retains 70% activity on the poly(dT) template, is the only one in which the poly(dA) synthesis activity has been dissociated extensively from the initiation reaction function. However, there is currently no direct evidence that Ad Pol is a zinc-binding enzyme or that the zinc-finger sequence is involved in DNA binding or polymerase function.

It appears from our initial linker-insertion mutagenesis that Ad Pol does not segregate its polymerase functions into definable subdomains of the entire polypeptide as occurs for the Klenow fragment of *E. coli* DNA polymerase I or for the murine reverse transcriptase (23–25). The evidence against segregated polymerase functions exclusively in either C- or N-terminal domains may be concluded from the inactive enzyme produced either by N-terminal deletion of 142 amino acids (22, 35) or by two inactive linker-insertion mutants (21A and 10A) with mutations located at the C terminus. In this respect, the Ad Pol resembles the herpes simplex virus DNA polymerase, for which mutants with altered reactivity to dNTP analogues map throughout the regions A, II, III, or V (44). Similar conclusions have been reached for the phage T4 DNA polymerase. T4 mutator-phenotype mutations have been mapped at sites far apart on the linear structure, but it has been proposed that these mutant sites may be close together on the folded, three-dimensional molecule (45). Recent evidence for the human immunodeficiency virus reverse transcriptase also indicates that the domains for polymerase and ribonuclease H activities are not completely distinct as they are for the murine retroviruses (46). Therefore, we favor a model in which some of the separate regions on the linear Ad Pol molecule interact to produce an active site for DNA binding, for dNTP binding, or for complex formation with other replication proteins. Because of these intramolecular interactions, such a model predicts that one should be able to isolate mutations in one Ad Pol region that can suppress mutations in other regions.

We are grateful to M. Scharff and J. Engler for their critical reading of the manuscript. The $pJ_1$-pol plasmid was obtained from J. Engler. The CMT4 line was kindly provided by Jeffrey Settleman (Yale University Medical School). This research was supported by Grants RO1 CA11512 and P30-CA13330 from the National Institutes of Health.

1. Robinson, A. J., Younghusband, H. B. & Bellett, A. J. D. (1973) *Virology* **56**, 54–69.
2. Rekosh, D. M. K., Russell, W. C., Bellett, A. J. D. & Robinson, A. J. (1977) *Cell* **11**, 283–295.
3. Lichy, J. H., Horwitz, M. S. & Hurwitz, J. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2678–2682.
4. Challberg, M. D. & Kelly, T. J., Jr. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 655–659.
5. Friefeld, B. R., Lichy, J. H., Field, J., Gronostajski, R. M., Guggenheimer, R. A., Krevolin, M. D., Nagata, K., Hurwitz, J. & Horwitz, M. S. (1984) *Curr. Top. Microbiol. Immunol.* **110**, 221–225.
6. Kelly, T. J., Jr. (1984) in *The Adenoviruses*, ed. Ginsberg, H. S. (Plenum, New York), pp. 271–308.
7. Lichy, J. H., Nagata, K., Friefeld, B. R., Enomoto, T., Field, J., Guggenheimer, R. A., Ikeda, J. E., Horwitz, M. S. & Hurwitz, J. (1983) *Cold Spring Harbor Symp. Quant. Biol.* **47**, 731–740.
8. Nagata, K., Guggenheimer, R. A. & Hurwitz, J. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 4266–4270.
9. Rosenfeld, P. J., O'Neill, E. A., Wides, R. J. & Kelly, T. J., Jr. (1987) *Mol. Cell. Biol.* **7**, 875–886.
10. Prujin, G. J. M., Van Driel, W. & vander Vliet, P. C. (1986) *Nature (London)* **322**, 656–659.
11. Guggenheimer, R. A., Nagata, K., Field, J., Lindenbaum, J., Gronostajski, R. M., Horwitz, M. S. & Hurwitz, J. (1983) *UCLA Symp. Mol. Cell. Biol.* **10**, 395–421.
12. Friefeld, B. R., Lichy, J. H., Hurwitz, J. & Horwitz, M. S. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 1589–1593.
13. Stillman, B. W., Tamanoi, F. & Mathews, M. B. (1982) *Cell* **31**, 613–623.
14. Lichy, J. H., Field, J., Horwitz, M. S. & Hurwitz, J. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 5225–5229.
15. Longiaru, M., Ikeda, J. E., Jarkovsky, Z., Horwitz, S. B. & Horwitz, M. S. (1979) *Nucleic Acids Res.* **6**, 1025–1040.
16. Challberg, M. D., Ostrove, J. M. & Kelly, T. J., Jr. (1982) *J. Virol.* **41**, 265–270.
17. Lindenbaum, J. O., Field, J. & Hurwitz, J. (1986) *J. Biol. Chem.* **261**, 10218–10227.
18. Field, J., Gronostajski, R. M. & Hurwitz, J. (1984) *J. Biol. Chem.* **259**, 9487–9495.
19. Horwitz, M. S. & Ariga, H. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 1476–1480.
20. Wong, S. W., Wahl, A. F., Yuan, P. M., Araz, N., Pearson, B. E., Araz, K. I., Korn, D., Hunkapiller, M. W. & Wang, T. S. F. (1988) *EMBO J.* **7**, 37–47.
21. Earl, P. L., Jones, E. V. & Moss, B. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 3659–3663.
22. Shu, L., Horwitz, M. S. & Engler, J. A. (1987) *Virology* **161**, 520–526.
23. Ollis, D. L., Brick, P., Hamlin, R., Xuong, N. G. & Steitz, T. A. (1985) *Nature (London)* **313**, 762–766.
24. Freemont, P. S., Ollis, D. L., Steitz, T. A. & Joyce, C. M. (1986) *Proteins Struct. Funct. Genet.* **1**, 66–73.
25. Tanese, N. & Goff, S. P. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 1777–1781.
26. Birnbaum, H. C. & Doly, J. (1979) *Nucleic Acids Res.* **7**, 1513–1523.
27. Gerard, R. D. & Gluzman, Y. (1985) *Mol. Cell. Biol.* **5**, 3231–3240.
28. Stone, J. C., Atkinson, T., Smith, M. & Dawson, T. (1984) *Cell* **37**, 549–558.
29. Lathe, R., Kienz, M. P., Skory, S. & Leweg, J. P. (1984) *DNA* **3**, 173–182.
30. Hoopes, B. C. & Maclure, W. R. (1981) *Nucleic Acids Res.* **9**, 5493–5504.
31. Gingeras, T. R., Sciaky, D., Gelinas, R. E., Bing-Dong, J., Yen, C. E., Kelly, M. M., Bullock, P. A., Parsons, B. L., O'Neill, K. E. & Roberts, R. J. (1982) *J. Biol. Chem.* **257**, 13475–13491.
32. Graham, F. L., Bacchetti, S., McKinnon, R., Stanners, C., Cordell, B. & Goodman, H. M. (1980) in *Introduction of Macromolecules into Viable Mammalian Cells*, eds. Baserga, R., Croce, C. & Rovera, G. (Liss, New York), pp. 3–25.
33. Friefeld, B. R., Korn, R., DeJong, P., Sninsky, J. J. & Horwitz, M. S. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 2652–2656.
34. Valderrama-Leon, G., Flomenberg, P. F. & Horwitz, M. S. (1985) *J. Virol.* **56**, 647–650.
35. Hassin, D., Korn, R. & Horwitz, M. S. (1986) *Virology* **155**, 214–224.
36. Stillman, B. W., Lewis, J. B., Chow, L. T., Mathews, M. B. & Smart, J. E. (1981) *Cell* **23**, 497–508.
37. Lyman, S. D. & Rohrschneider, L. R. (1987) *Mol. Cell. Biol.* **7**, 3287–3296.
38. Giguere, U., Hollenberg, S. M., Rosenfeld, M. G. & Evans, R. M. (1986) *Cell* **46**, 645–652.
39. Ng, M. & Privalsky, M. L. (1986) *J. Virol.* **58**, 542–553.
40. Wilkie, N. J., Ustacelebi, S. & Williams, J. F. (1973) *Virology* **51**, 499–503.
41. Miller, B. W. & Williams, J. (1987) *J. Virol.* **61**, 3630–3634.
42. Miller, J., McLachlan, A. D. & Klug, A. (1985) *EMBO J.* **4**, 1609–1614.
43. Berg, J. M. (1986) *Science* **232**, 485–487.
44. Gibbs, J. S., Chiou, H. C., Bastow, K. F., Cheng, Y. C. & Coen, D. M. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 6672–6676.
45. Reha-Krantz, L. J. (1988) in *DNA Replication and Mutagenesis*, eds. Moses, R. B. & Summers, W. C. (Am. Soc. Microb., Washington, DC), pp. 34–40.
46. Prasad, V. R. & Goff, S. P. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 3104–3108.