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Mutations in Two Cysteine-Histidine-Rich Clusters in Adenovirus Type 2 DNA Polymerase Affect DNA Binding

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Several point and linker insertion mutations in two Cys-His-rich regions of adenovirus (Ad) DNA polymerase (Pol) gene have been expressed in recombinant vaccinia virus. The resulting mutant enzymes were analyzed in vitro for their effects on DNA synthesis activity, on Ad-specific initiation assays, on gel shifts of Ad origin sequences, and on interactions with adenovirus preterminal protein (pTP) and nuclear factor I (NFI). In general, mutants in downstream Cys-His sequences had a pronounced effect in these assays. Mutants in the upstream Cys-His region had a moderate effect on DNA synthesis and elongation but failed to make dCMP-pTP initiation complexes and failed to make specific shifted complexes in a gel retardation assay. These mutants could still bind to pTP and NFI in a coimmunoprecipitation experiment, suggesting that this upstream Cys-His region of Ad Pol is involved either in specific Ad DNA origin binding or in nonspecific DNA binding. Changing residues within Cys doublets in the downstream Cys-His region had pronounced effects on many Ad Pol functions such as DNA synthesis, DNA binding, and in vitro initiation; however, these mutants showed little reduction in binding to pTP and NFI; mutants at other cysteines or histidines within this region of Ad Pol did not appear to have an effect on enzyme function. This observation suggests that the downstream Cys-His region of Ad Pol is important for DNA binding and might fold into a Zn finger motif.

The replication of the 36,000-bp adenovirus (Ad) genome is initiated by a protein priming mechanism, in which the virus-encoded precursor to the terminal protein (pTP) is covalently attached to dCTP, the first nucleotide found at each 5′ end of the linear Ad DNA. The Ad DNA polymerase (Ad Pol) is a second essential component in the process of Ad DNA replication and is a 140-kDa polypeptide encoded from the leftwardly transcribed strand in the viral E2B region between genome coordinates 24 and 14. Ad Pol is absolutely required for efficient initiation as well as for elongation of the replication fork during subsequent DNA synthesis. A third virus protein, the single-stranded DNA-binding protein (DBP), stabilizes DNA strands displaced by the movement of Ad Pol along the DNA molecule. Three host factors (NFI, NFII, and NFIII) are also required for efficient replication in vitro. Replication complexes consisting of Ad Pol, pTP, NFI, and NFIII form at origins located at each end of the Ad genome (for reviews, see references 7, 14, and 42).

Eukaryotic DNA replication is a complex process which requires the orderly and precise interplay between DNA and a variety of different proteins; since the initial description of an in vitro system for Ad DNA replication by Challberg and Kelly (6), this system has provided a useful paradigm for the study of eukaryotic DNA replication. For example, Ad Pol is a member of the polymerase α family of DNA polymerases; Ad Pol shares five of six conserved regions with other members of this family of polymerases (47). During DNA replication, DNA polymerase binds to DNA, to nucleotide triphosphates, and to other required protein factors. Ad Pol provides a good model system for studying the role of different functional domains in DNA polymerases because of the well-characterized in vitro assays for the many steps required in Ad DNA replication and because of the availability of several transient expression systems, such as transfected plasmids (37), vaccinia virus (32, 40), and baculovirus (49), that can be used to overproduce this polymerase. Information provided from studying mutants of Ad Pol may be extended by analogy to other eukaryotic DNA polymerases.

Previous studies indicated that the mutations in conserved region I (which is located near the C terminus of Ad Pol) resulted in proteins that were inactive for many DNA synthesis and replication functions, perhaps as a result of defects in substrate binding (21). Other conserved regions have also been proposed to be substrate or metal ion binding sites, as indicated by studies of the herpes simplex virus DNA polymerase (12, 28) and of the 3′-5′ exonuclease site in the bacteriophage ϕ29 DNA polymerase (5). Domains within Ad Pol that are required for binding to Ad DNA or to pTP have not yet been precisely defined; however, Chen et al. (11) have begun to map the domains required for interaction of Ad Pol and NFI.

Protein-DNA recognition is often mediated by a small domain containing a structural motif, such as a helix-turn-helix or a zinc finger. A zinc finger motif usually consists of doublets of Cys (CXXC) separated by approximately 12 amino acids from doublets of His; in some cases, a second Cys doublet can substitute for the His doublet (4). It has been suggested that this sequence binds a zinc ion through the invariant Cys and His residues to form a domain that is important for protein-protein interactions or for DNA binding (2–4, 25). In Ad Pol, there are two Cys-His-rich sequences that are conserved between different Ad serotypes and that might be folded into zinc finger motifs that bind to DNA (10); these sequences do not precisely fit the consensus for a zinc finger (Fig. 1A). The first Cys-His-rich sequence is located between amino acids 228 and 256 and is composed of the sequence CQYC(amino acids)$_{20}$HINSH. A mutant containing a four-amino-acid insertion between the first two cysteines in this upstream sequence failed to bind Ad DNA

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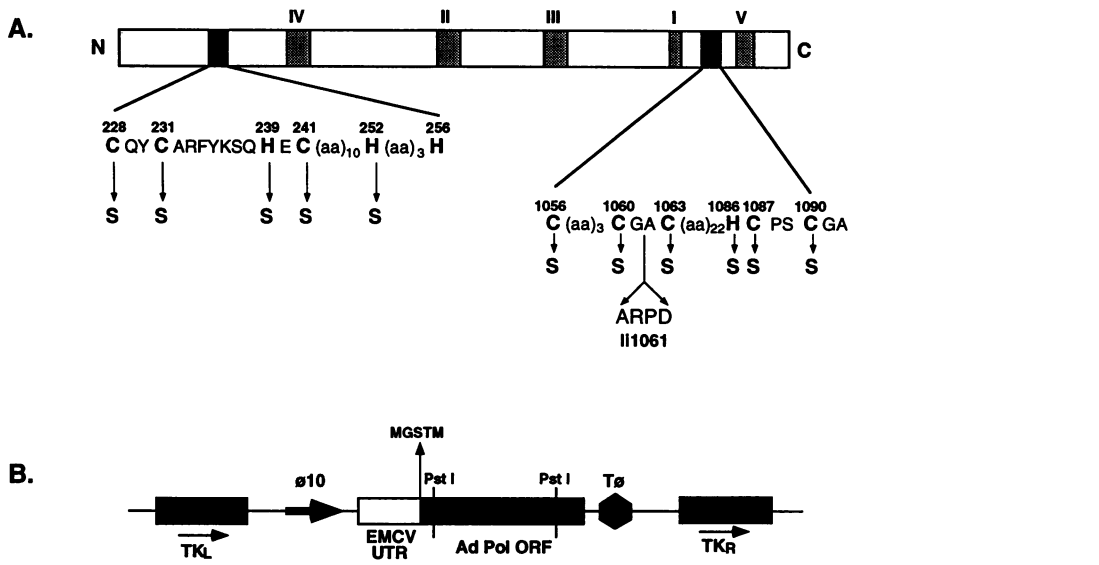

FIG. 1. (A) Locations of Cys-His-rich regions in Ad Pol and position of mutations. The Ad Pol ORF is represented as a linear molecule from amino (N) to carboxyl (C) termini; both Cys-His-rich regions are shown as filled black rectangles, with the sections between amino acids (aa) 228 to 256 and amino acids 1056 to 1090 expanded to show amino acid sequence; the numbers above the amino acids correspond to their positions in the deduced protein sequence for Ad Pol (with amino acid 4 starting at nucleotide 8473 [38]). The left-hand region has a Cys doublet at amino acids 228 and 231 and a His doublet at 252 and 256; the right-hand region has two Cys doublets at amino acids 1060, 1063, 1087, and 1090. Stippled boxes represent conserved regions (region I to V) found in the DNA polymerase α family (47). The cysteines and histidines that were each replaced with serine are indicated by arrows, and the deduced sequence created by the linker insertion mutation at amino acid 1061 (*li*1061) is shown below the position of the inserted amino acid sequence. (B) Schematic diagram of the vaccinia virus expression vector pTEAP2 for expressing Ad Pol. The *Bam*HI fragment encoding Ad Pol ORF (indicated by a black bar) was fused to the EMCV UTR (indicated by an open bar) in frame by blunt-end ligation. As a result of this ligation, the N-terminal sequence of Ad Pol was changed from MAL to MGSTM. The EMCV UTR-Ad Pol chimeric gene is placed behind the bacteriophage T7 promoter ø10 (black arrow) and flanked by the left (TK_L) and the right (TK_R) vaccinia virus TK gene sequences (16). Each mutated DNA was exchanged with the corresponding wt fragment by digestion with *Pst*I (nucleotides 8397 to 4964).

origin sequences but could still interact with other proteins required in Ad DNA replication in vitro, suggesting that this sequence might encode a DNA binding domain of Ad Pol (11). A second region is located between conserved regions I and V (47) near the C terminus of Ad Pol and includes conserved cysteines at residues 1060, 1063, 1087, and 1090 (CGACGC(amino acids)$_{23}$CPSC).

In this investigation, we examined whether the N-terminal and C-terminal Cys-His-rich sequences are important in Ad Pol function and, if so, whether these sequences are involved in DNA binding or in interations with other proteins. For these studies, each cysteine and histidine residue in these two regions of Ad Pol was changed to serine; in addition, one linker insertion mutation that altered the spacing between cysteines in the C-terminal sequences was studied. Each mutated Ad Pol gene was expressed by using the vaccinia virus/T7-encephalomyocarditis virus (EMCV) hybrid system; the resulting mutant proteins were analyzed for their effects on DNA synthesis activity, on DNA binding, on in vitro Ad initiation, and on interactions with pTP and with NFI.

MATERIALS AND METHODS

Plasmids and mutagenesis. The sequence immediately upstream of the splice acceptor site for Ad Pol in pAP-118 (the Ad Pol open reading frame [ORF] cloned in pUC118 [46]) was changed to 5′-TGGATCCACAATGGTTCAAGCT-3′ by site-directed mutagenesis, to create a *Bam*HI cleavage site and translation initiation codon for Ad Pol. Plasmid pE5AP was constructed by making the *Bam*HI fragment (containing the Ad Pol ORF) from this plasmid blunt ended with the Klenow enzyme and cloning the fragment behind a bacteriophage T7 promoter-EMCV untranslated region (UTR) in pE5LVPØ (34), which had been previously cleaved with *Bal*I and *Xba*I and filled in with the Klenow enzyme. Expression vector pTEAP2 (Fig. 1B) was constructed by inserting an *Eco*RI-*Sal*I fragment from pE5AP (filled in to make blunt ends) into a cleaved and filled-in *Bam*HI site between the bacteriophage T7 promoter (ø10) and terminator (Tø) in plasmid pTF7-3 (16). This plasmid (pTEAP2) contained the coding sequence for Ad Pol downstream of the T7 promoter and EMCV UTR; this chimeric gene was flanked by the left (TK_L) and right (TK_R) vaccinia virus thymidine kinase (TK) gene sequences for homologous recombination with wild-type (wt) vaccinia virus (Fig. 1B).

Single amino acid replacement mutants were generated in two regions, amino acids 228 to 256 and 1056 to 1090, in Ad Pol by site-directed mutagenesis using pAP-118 (51). Mutant *li*1061 was made by insertion of a 12-bp *Bam*HI linker (5′-CCCGGATCCGGG-3′) at *Hae*III sites within the Ad Pol ORF in pAP-118-ΔBam (in which the unique *Bam*HI site in the polylinker of pAP-118 was removed). Linker mutagenesis was performed as described by Barany (1), and the site of insertion was determined by restriction enzyme digestion and by double-stranded DNA sequencing (29).

The amino acids changed in these sequences were shown in Fig. 1A. Ad Pol DNA containing the mutations was cloned into pTEAP2 by exchanging the *Pst*I fragment (nucleotides 8397 to 4964) of the mutated Ad Pol ORF with that of the wt;

once the mutations were cloned into pTEAP2, the presence of each mutation was reconfirmed by double-stranded DNA sequencing.

Plasmid pE7NF-I was constructed for in vitro expression of NFI. The *Nco*I-*Sal*I fragment of pCTF-I (37) was cloned into pE5LVPØ (that had also been cut with *Nco*I and *Sal*I), placing the coding sequence for NFI downstream of a bacteriophage T7 promoter and the EMCV UTR.

Cell culture and construction of recombinant vaccinia viruses. African green monkey kidney (CV-1) cells and human epidermoid carcinoma (HEp-2) cells were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO Laboratories) with 10 and 2.5% fetal calf serum (HyClone), respectively. HeLa cells were grown in DMEM with 5% bovine calf serum (HyClone), and human TK^- 143 cells (36) were grown in DMEM with 10% bovine calf serum in the presence of 25 μg of bromodeoxyuridine (BUdR) per ml.

Recombinant viruses were prepared by infection of a 100-mm-diameter plate of CV-1 cells with wt vaccinia virus for 1 h, after which 20 μg of plasmid pTEAP2 containing various mutations was transfected by the calcium phosphate precipitation method (9). After 24 h, the cells were harvested and TK^- recombinant viruses were isolated by plaque assay on human TK^- 143 cells in the presence of 25 μg of BUdR per ml. The virus producing Ad Pol (vTEAP2) was identified by screening for expression of the 140-kDa polymerase on a Western immunoblot. Virus was then plaque purified in TK^- cells with BUdR selection, and large stocks of recombinant virus were prepared under nonselective conditions in HEp-2 cells.

Protein expression and preparation of cell lysates. One-hundred-millimeter-diameter plates of HEp-2 cells were coinfected with vTEAP2 and a second recombinant vaccinia virus which produces T7 RNA polymerase (vTF7-3 [16]; multiplicity of infection of 10 for each virus), and cell lysates were prepared at 20 h postinfection. For in vitro binding assays, cells were extracted in lysis buffer (50 mM Tris-Cl [pH 8.0], 250 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA) for 10 min on ice and then centrifuged at 10,000 × *g* in an Eppendorf microcentrifuge for 15 min at 4°C to clear the lysate of debris. For other assays, crude cytoplasmic extracts were prepared in hypotonic buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.5], 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol) by Dounce homogenization (6). The amount of 140-kDa Ad Pol protein in each mutant extract was quantitated as described previously (21) by densitometry of a Western blot developed by using a rabbit polyclonal antiserum generated against an Ad Pol peptide (amino acid codons 1007 to 1021; KSVYGDTDSLFVTERC). To determine relative amounts of each mutant protein, known amounts of a wt Ad Pol extract were included in each Western blot used for quantitation; in this way, the amounts of protein included in the quantitation could be kept in the linear range of the densitometer and the amounts of Ad Pol mutant proteins could be compared directly with known amounts of wt Ad Pol extract. The estimated error for this quantitation is approximately in the 20% range because of the variations in amounts of expressed proteins in each time and technical variations of the densitometer. In each subsequent assay, the same amount of Ad Pol protein was added, as determined by this quantitation.

Replication proteins. Ad DBP was prepared from Ad2-infected HeLa cells (20), and NFI was prepared from uninfected HeLa cells as described previously (31). pTP was prepared from cells infected with recombinant vaccinia virus containing the pTP gene (14a), and a crude cytoplasmic extract was used for assays.

Labeling of pTP and NFI. A 100-mm-diameter plate of HEp-2 cells coinfected with vaccinia viruses producing pTP and vTF7-3 was incubated for 1 h in methionine-free DMEM at 20 h postinfection and then labeled with 250 μCi of [^{35}S]methionine (1,000 Ci/mmol; Amersham) for 1 h at 37°C. Cells were extracted with 2 ml of lysis buffer and used for the binding assay after removal of cell debris. The NFI RNA was synthesized in vitro from plasmid pE7NF-1 by using bacteriophage T7 RNA polymerase, and the resulting RNA was translated in vitro by using a rabbit reticulocyte lysate in the presence of [^{35}S]methionine as instructed by the manufacturer (Stratagene, Inc.). This crude translated mixture was used for the NFI binding assay.

DNA polymerase assays. (i) DNA synthesis assay. Aphidicolin-resistant Ad Pol activity in each cytoplasmic extract was determined by measuring the incorporation of [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham) into a nicked calf thymus DNA template in the presence of 120 μM aphidicolin (39); the reaction mixture was incubated for 60 min at 30°C, the reaction was terminated by adding 1/10 volume of 0.1 M EDTA (pH 8.0), and the mixture was then kept on the ice for 15 min. Radioactivity was determined by scintillation counting as described previously (21). This assay was used to measure the relative activity of Ad Pol or its mutants for general DNA synthesis on this nicked template.

(ii) Ad Pol elongation assay. Ad-specific DNA polymerase activity was measured by using a poly(dT)-oligo(dA) template-primer (Pharmacia) in the presence of Ad DBP and [α-^{32}P]dATP (3,000 Ci/mmol; Amersham) as described previously (14); other contaminating cellular DNA polymerases in these crude extracts have little or no activity on this template. Because this assay requires both Ad Pol and DBP for activity, it has been suggested that this assay can be used to measure Ad DNA elongation in a reaction that is not dependent on prior initiation of DNA replication with pTP and Ad origin DNA (14). The assay reaction mixture was incubated for 60 min at 37°C prior to termination of the reaction and measurement of the incorporated radioactivity as described above.

In vitro assay for initiation of Ad DNA replication. The activity of mutant Ad Pol in each cytoplasmic extract to initiate DNA replication was measured by formation of a ^{32}P-labeled pTP-dCMP complex, using synthetic duplex template TD16 which contains Ad DNA end sequences as described previously (23).

In vitro assay for pTP and NFI binding. The coimmunoprecipitation assay used to test binding of pTP and NFI to Ad Pol is similar to one described by Chen et al. (11). One hundred microliters of labeled pTP extract and various amounts of unlabeled Ad Pol mutant extract were mixed in 600 μl of lysis buffer and incubated for 2 h on ice. Ten microliters of in vitro-translated and labeled NFI was mixed with various amount of unlabeled Ad Pol mutant extract in 600 μl of buffer (25 mM HEPES [pH 7.5], 50 mM $MgCl_2$, 5 mM dithiothreitol, 200 mM NaCl), and the reaction mixture was incubated for 2 h at 30°C. After incubation, 2 μl of a polyclonal antibody against a β-galactosidase–Ad Pol fusion protein (kindly provided by R. Padmanabhan) was added, and the mixtures were rocked overnight. The antibody-protein complex was precipitated by using protein A-Sepharose beads (Pharmacia), boiled in protein loading (cracking) buffer (26), and separated by electrophoresis on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel. Gels were

dried after fixation and treatment with En^{3}Hance (NEN Research Products) and exposed to X-ray film at −70°C.

Gel retardation assay for DNA binding. For the DNA binding assay, a partially duplex oligonucleotide, TD16 (23), which contains Ad DNA end sequences was used as described previously, with the following modification. Three picomoles of an oligonucleotide that contained the first 50 bp of the Ad template strand was labeled at its 5′ end with T4 polynucleotide kinase (Pharmacia) and [γ-^{32}P]rATP (3,000 Ci/mmol; Amersham) and was annealed with a 10-fold excess of a 35-bp oligonucleotide that contained nucleotides 16 to 50 of the displaced strand of the Ad origin. Annealing was carried out by heating at 65°C for 5 min, followed by slow cooling to room temperature. Crude pTP (2.5 μg) and wt or mutant Ad Pol extracts and 6 μg of poly(dI-dC) were preincubated for 10 min on ice in 20 μl of binding buffer (20 mM Tris-Cl [pH 7.5], 50 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 8% Ficoll). Then 60 fmol of end-labeled and annealed TD16 was added to the mixture, which was incubated for an additional 20 min on ice. Half of each reaction mixture was loaded on 5% nondenaturing polyacrylamide gel (82:1 acrylamide/bisacrylamide) and run for 75 min at 13 V/cm in 0.5× TBE buffer (0.045 M Tris-borate, 0.001 M EDTA). The gels were then dried and exposed to X-ray film at −70°C.

RESULTS

Construction of recombinant vaccinia virus. To increase the level of expressed protein, the Ad Pol ORF was placed behind the EMCV untranslated sequence so that the EMCV major translational start signal was used; inclusion of this leader sequence improves the translation efficiency of uncapped mRNAs (15, 45). During the cloning of vaccinia virus expression vector pTEAP2, the N terminus of Ad Pol was changed from MAL to MGSTM during ligation to the EMCV DNA fragment. Protein was made by coinfecting HeLa cells with a recombinant vaccinia virus containing the Ad Pol gene (vTEAP2; Fig. 1B) and a recombinant virus which produces bacteriophage T7 RNA polymerase (vTF7-3); the resulting enzyme produced a 140-kDa band on a Western blot developed with antipeptide antibodies specific for Ad Pol and was fully active in DNA synthesis and in initiation assays used in this study (data not shown); the change in N-terminal amino acid sequence had no apparent effect on the activity of wt Ad Pol. When the amount of protein expressed in this system was compared with that of other recombinant vaccinia virus systems in which the Ad Pol gene was placed behind the vaccinia virus late promoter p11 (20a, 40), the vaccinia virus/T7-EMCV system made at least five times more protein (Fig. 2). The expression level observed for the vTEAP2 virus was constant during growth of virus stocks, indicating that the Ad Pol gene was stably inserted into this vaccinia virus genome; on the other hand, virus vIJAP (a vaccinia virus recombinant with the Ad Pol gene under control of the p11 promoter) showed a propensity to delete Ad Pol sequences when propagated (unpublished observation).

Construction of mutations in Cys-His-rich sequences. Every cysteine and histidine codon in the regions between amino acids 228 to 256 and between amino acids 1056 to 1090 of Ad Pol was mutated to test whether these sequences are important for protein function. Serine was chosen to replace Cys or His in order to minimize structural distortion; the resulting mutants are listed in Fig. 1A. In addition, the position of one linker insertion mutant at a *Hae*III site (nucleotide 5613) was shown to be located in Cys-His-rich sequences by DNA sequencing; this mutant increased the spacing between two cysteines at amino acids 1060 and 1063 from two to six amino acids (*li*1061; CGARPDAC).

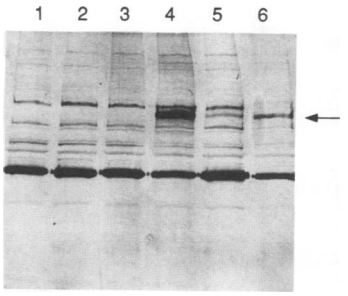

FIG. 2. Comparison of the Ad Pol expression level in a vaccinia virus/T7-EMCV hybrid system with those in other recombinant vaccinia virus systems. Protein samples were prepared from infected HEp-2 cells as described in Materials and Methods; identical amounts of total protein in all extracts were analyzed by SDS-PAGE on a 7.5% gel and then subjected to immunoblot analysis using a rabbit polyclonal antiserum raised against a synthetic peptide sequence (KSVYGDTDSLFVTERC) from region I of Ad Pol. The expected size of Ad Pol is indicated by an arrow. Lanes: 1, wt vaccinia virus infected; 2, vTF7-3 infected; 3, wt vaccinia virus and vTF7-3 coinfected; 4, vTEAP2 and vTF7-3 coinfected; 5, vIJAP infected; 6, vVVAP infected. The recombinant virus vIJAP contained the vaccinia virus late promoter p11 fused to the Ad Pol gene and the *Escherichia coli gpt* gene driven by the vaccinia virus promoter p7.5 (13) and was recombined into the *tk* locus (unpublished data). The recombinant virus vVVAP (39) had the Ad Pol gene placed behind a mutated p11 promoter, which presumably increases synthesis of foreign polypeptides during the late phase of virus infection.

The total amounts of expressed Ad Pol protein in various infected cell extracts were somewhat variable. To compare the activities of mutated proteins, the relative amounts of mutant Ad Pol proteins in crude cytoplasmic extracts were determined by quantitative Western blotting (Fig. 3). These mutant Ad Pol enzymes were tested for susceptibility to proteolytic degradation when expressed in transfected and infected HEp-2 cells in order to rule out the possibility that these mutations merely increased protein turnover. Increased proteolytic degradation might be expected for these mutants if the proteins are aberrantly folded. In pulse-chase experiments (not shown), HEp-2 cells were coinfected with vTF7-3 and vTEAP2 containing wt or mutated Ad Pol genes. At 20 h postinfection, infected cells were transferred to methionine-deficient medium and labeled for 1 h with [^{35}S]methionine (pulse experiments), followed by chases of up to 4 h with added unlabeled methionine. Each mutant Ad Pol showed little apparent loss of label in a 140-kDa band on an SDS-polyacrylamide gel when the intensities of pulsed and chased material were compared (data not shown).

Effects of mutations on DNA polymerase activity. The DNA synthesis activity of each mutant was tested in two assays. The first assay used nicked calf thymus DNA as a template to measure general DNA synthesis activity; Ad Pol activity can be distinguished from that of endogenous cellular DNA polymerases by the inclusion of 120 μM aphidicolin in this assay (39). Only the four cysteine changes (C1060S, C1063S, C1087S, and C1090S) and the linker insertion (*li*1061) in the C-terminal sequence showed decreased activity; mutants at other sites showed little reduction in activity in this assay (Table 1).

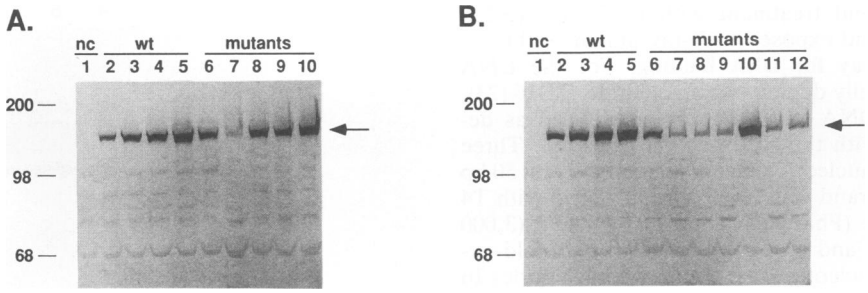

FIG. 3. Western blot analysis to quantitate the amount of Ad Pol in extracts containing various mutated Ad Pol proteins. Increasing amounts (5, 10, 15, and 20 μg) of a cytoplasmic extract containing wt Ad Pol (lanes 2 to 5 in panels A and B) and 20 μg of extracts from cells infected with vaccinia viruses expressing the mutated Ad Pol genes were loaded on the same SDS-polyacrylamide gel. (A) Lane 6, C228S; lane 7, C231S; lane 8, H239S; lane 9, C241S; lane 10, H252S. (B) Lane 6, C1056S; lane 7, *li*1061; lane 8, C1060S; lane 9, C1063S; lane 10, H1086S; lane 11, C1087S; lane 12, C1090S. Western blotting was performed as described in Materials and Methods, and the amount of Ad Pol in each lane was determined by densitometry of the Ad Pol band, as indicated by arrows. A standard curve was prepared by plotting the density (after subtracting the density of the negative control in lane 1) of the wt Ad Pol band versus total protein concentration in extracts. By using this standard curve, the relative amount of Ad Pol protein in each mutant extract was determined. These normalized amounts were used to determine the volume of each extract to be added in the subsequent assays for enzyme activity in order to ensure that equal amounts of wt or mutant Ad Pol proteins were included in all reactions. Sizes are indicated in kilodaltons.

In the second assay, poly(dT)-oligo(dA) was used as a template-primer and Ad Pol-specific DNA elongation activity was determined in a reaction that also required Ad DBP and [α-^{32}P]dATP. Mutants in the C-terminal sequence also showed pronounced effects on observed activity in this elongation assay. Mutants at the cysteine at 1056 and at the histidine at 1086 (which are not located in one of the Cys or His doublets) showed activities comparable to the wt level of DNA synthesis. In the N-terminal Cys-His region, the mutants at the first cysteines (C228S and C231S) and at the following Cys (C241S) showed slightly decreased activity, but the mutants at His-239 and at His-252 were as active as the wt in this assay (Table 1).

TABLE 1. DNA polymerase activities in cytoplasmic extracts from cells infected with various mutants

Virus	Sp act[a]	
	Nicked DNA[b]	dT-dA[c]
Control[d]	1.7	0.9
wt	53.5	64.6
C228S	45.4	32.9
C231S	49	21.7
H239S	81.2	46.2
C241S	56.5	39.5
H252S	59.8	67
C1056S	58	53
C1060S	19	16
*li*1061	22	8
C1063S	19.5	18
H1086S	59.5	57.4
C1087S	22	21
C1090S	23	16

[a] Specific activities of DNA polymerase were defined as picomoles of nucleotides incorporated into the templates for 60 min by 1 μg of total protein in cytoplasmic extracts from cells infected with various mutant viruses. These numbers were then normalized (shown in the table) by relative amounts of Ad Pol on the basis of the quantitation. Estimated error for relative Ad Pol quantitation is approximately ±50%. Data from three independent assays have been averaged.

[b] DNA polymerase activity was measured by the incorporation of dCTP in the presence of 120 μM aphidicolin. Cytoplasmic extracts equivalent to 5 μg of total protein were added to 25 μl of reaction mixture containing 200 μg of nicked calf thymus DNA per ml and 40 μM deoxynucleoside triphosphates.

[c] DNA polymerase activity was measured by the incorporation of dATP in the presence of Ad DBP; 20 μl of reaction mixture contained 66 μg of dT-dA per ml and 0.8 μM dATP.

[d] The cytoplasmic extract from the cells infected with only vTF7-3 was used for the control experiment.

Effects on the initiation reaction. In the initiation reaction, Ad Pol activity was assayed by the transfer of label from [α-^{32}P]dCTP to an 80-kDa pTP-dCMP complex, as detected on an SDS-gel. The effects of different mutants on Ad Pol protein function in this assay were rather interesting, because the mutants in the left region (cysteines at 228, 231, and 241 and histidine at 239) did not form initiation complexes, even though they were still active in DNA elongation reactions, suggesting that this region might be involved in other interactions. The effects exhibited by mutants in the C-terminal region were also more pronounced (Fig. 4). Mutants C1056S and H1086S had wt-like activity, whereas mutants at all other sites resulted in inactive proteins, consistent with the results observed in the previous DNA synthesis assays.

Effects on pTP and NFI binding. In the initiation assay, interactions of Ad Pol with Ad origin DNA, with pTP, and with NFI are all required to make a pTP-dCMP initiation complex. To test whether the apparent defect in the initiation assays was due to inability of Ad Pol mutants to bind to pTP or to NFI, coimmunoprecipitation assays to measure binding of Ad Pol to pTP and to NFI were used. These assays measured the ability of [^{35}S]methionine-labeled pTP or NFI to form a complex with unlabeled wt or mutant Ad Pol proteins; the putative complexes were then precipitated with an antibody directed against Ad Pol, and the appearance of a labeled pTP (Fig. 5A) or NFI (Fig. 5B) band was detected on an SDS-polyacrylamide gel. In the right Cys-His region, mutants C1060S, C1063S, C1087S, and C1090S, which were completely inactive in the initiation assay, were either as good as the wt or only slightly decreased in their ability to bind to pTP. pTP binding to mutants in the upstream Cys-His region (Fig. 5A and Table 2) was virtually the same as that to wt Ad Pol, indicating that the defect of mutants in two Cys-His regions in the initiation reaction was not due to dramatic changes in binding of the Ad Pol mutants to pTP. Similar results were observed for NFI binding to Ad Pol; mutants in both the upstream and downstream regions still bound to NFI at wt or nearly wt levels (Fig. 5B and

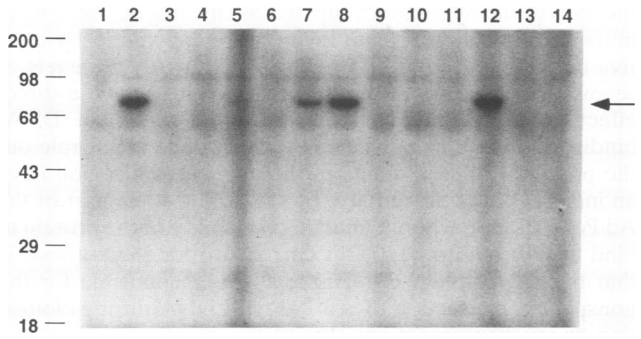

FIG. 4. Initiation assays for determination of the activity of Ad Pol and its mutants. This assay measures the covalent attachment of [α-^{32}P]dCMP to pTP in an in vitro reaction that is also dependent on Ad Pol, Ad DNA-protein complex, and NFI. The position expected for labeled 80-kDa pTP is indicated by an arrow. Wild-type and mutant Ad Pol extracts were made by coinfecting the cells with vTF7-3 and the specific Ad Pol recombinant virus listed below; cellular extract from HEp-2 cells infected only with virus vTF7-3 was used as a negative control. Equal amounts of Ad Pol protein (as determined by quantitative immunoblotting in Fig. 3) were used. Mutants in left and right Cys-His-rich regions are shown in lanes 3 to 7 and 8 to 14, respectively. Lanes: 1, vTF7-3 only; 2, wt; 3, C228S; 4, C231S; 5, H239S; 6, C241S; 7, H252S; 8, C1056S; 9, *li*1061; 10, C1060S; 11, C1063S; 12, H1086S; 13, C1087S; 14, C1090S. Sizes are indicated in kilodaltons.

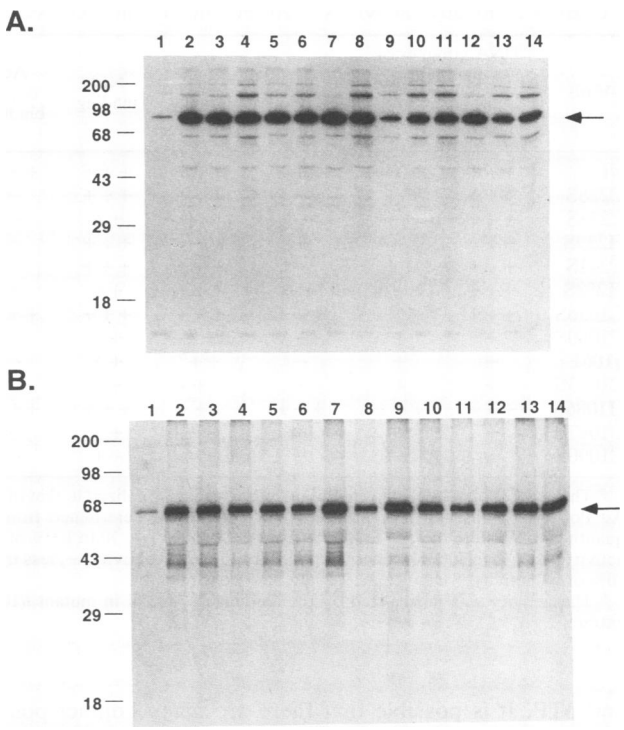

FIG. 5. Binding of pTP and NFI to Ad Pol and its mutants. Labeled pTP and NFI were reacted with wt or mutant Ad Pol extracts in vitro and coimmunoprecipitated with anti-Ad Pol antibody as described in Materials and Methods. The resulting precipitates were analyzed by SDS-PAGE on a 10% gel. In panels A and B, there is a faint nonspecific background band which appears at the position of pTP or NFI. (A) pTP binding of Ad Pol mutants in Cys-His regions. Lanes 3 to 7 and 8 to 14 show mutations in the left and right Cys-His-rich regions, respectively. The arrow shows the expected position for the 80 kDa pTP protein. In lane 9, the amount of pTP which can be coimmunoprecipitated is lower because of a lower relative amount of mutant Ad Pol added; when normalized for the amount of mutant Ad Pol protein, the amount of pTP coimmunoprecipitated is nearly the same as for the wt (Table 2). Lanes: 1, negative control (vTF7-3 only); 2, wt; 3, C228S; 4, C231S; 5, H239S; 6, C241S; 7, H252S; 8, C1056S; 9, *li*1061; 10, C1060S; 11, C1063S; 12, H1086S; 13, C1087S; 14, C1090S. (B) NFI binding of Ad Pol mutants. The order of the samples is same as in panel A. The arrow shows the expected position for NFI. In lane 8, the amount of NFI which can be coimmunoprecipitated is lower because of a lower relative amount of mutant Ad Pol added; when normalized for the amount of mutant Ad Pol protein, the amount of NFI coimmunoprecipitated is nearly the same as for wt Ad Pol (Table 1). Sizes are indicated in kilodaltons.

Table 2). In both assays, a small but reproducible amount of nonspecific precipitation of pTP or NFI was observed (Fig. 5A and B, lanes 1).

Effects on DNA binding. The binding of mutant Ad Pols to Ad origin DNA was checked by using a partially duplex oligonucleotide, TD16 (23), which contained 50 bp of origin sequence from the Ad template strand and 35 bp from the displaced strand. The Ad Pol-pTP complex, bound to this partially single-stranded Ad DNA fragment, made a specific shifted complex (Fig. 6A, lane 3), whereas binding by pTP or by Ad Pol alone did not result in detectable shifted bands (Fig. 6A, lanes 9 and 10). In addition, several nonspecific band shifts were also apparent in these extracts. The ability of mutant Ad Pols to produce a gel shift on TD16 was then investigated. The mutations in the left Cys-His region showed decreased ability to bind TD16 in the gel; extracts from mutants C228S, C231S, H239S, and C241S made little or no detectable shifted complex, while mutant H252S showed reduced ability to shift TD16. This observation was in agreement with the results obtained in the initiation reaction. In the right Cys-His sequence, mutants C1056S and H1086S had little effect on the ability to form specific retarded complexes, but other mutant proteins were unable to shift TD16 (Fig. 6B); these observations are in accordance with the results of the other assays. Within the C-terminal Cys-His region, cysteines located in Cys doublets appear to be important in DNA binding of Ad Pol.

DISCUSSION

The results presented above suggest that each of the two Cys-His clusters in Ad Pol may be involved in the DNA binding, but that each of these regions may play a different role in Ad replication; mutants in the downstream Cys-His region were defective in most assays, while mutants in the upstream region appeared to be functional in DNA synthesis and elongation assays but not in Ad-specific initiation assays (Table 2). These results concur with previous conclusions based on the analysis of linker insertion mutants of Ad Pol (10, 11); except for the mutants in the upstream Cys-His region, most mutations in Ad Pol appear to be either entirely permissive or nonpermissive in all in vitro assays currently available (with the exception of coimmunoprecipitation assays with pTP and with NFI). This observation suggests that domains within Ad Pol are very interdependent: alterations in one domain may have dramatic effects on other domains within the protein. A similar conclusion has been drawn for the herpes simplex virus DNA polymerase on the basis of analysis of drug resistance and hypersensitivity mutants (28, 48). Surprisingly, mutants in each of the two Cys-His regions appear to be nearly wt in their ability to interact with NFI

TABLE 2. Relative activities of Ad Pol mutants in each assay[a]

Virus	DNA polymerase activity		Initiation	pTP binding	NFI binding	Ad origin binding
	Nicked DNA	dT-dA				
wt	+++	+++	+++	+++	+++	+++
C228S	+++	++	−	+++	+++	−
C231S	+++	++	−	++	++	−
H239S	+++	+++	+	+++	+++	−
C241S	+++	++	−	+++	+++	−
H252S	+++	+++	++	+++	++	+
C1056S	+++	++	+++	+++	+++	+++
C1060S	+	+	−	++	++	−
*li*1061	+	+	−	++[b]	++	−
C1063S	+	+	−	+++	++	−
H1086S	+++	+++	+++	+++	+++	+++
C1087S	+	+	−	++	++	−
C1090S	+	+	−	++	++	−

[a] The activity of Ad Pol in each mutant extract was normalized to that of wt Ad Pol on the basis of the amount of Ad Pol protein determined from a quantitative Western blot, so that wt activity is 100%. +++, 70 to 100% of wt activity; ++, 40 to 70% of wt activity; +, 10 to 40% of wt activity; −, less than 10% of wt activity.

[b] The activity was normalized by the amount of Ad Pol in mutant *li*1061 extract.

and pTP. It is possible that there are many contact points between NFI and Ad Pol and between pTP and Ad Pol and that mutants at a particular site in Ad Pol may disrupt one contact with these proteins without affecting the other binding sites.

Although there is no evidence that Ad Pol binds zinc, mutants in each of these regions behaved as expected for a zinc finger domain; (i) mutants that altered conserved cysteines or histidines within the zinc finger motif (Cys-228, Cys-231, His-252, Cys-1060, Cys-1063, Cys-1087, and Cys-1090) showed greatly reduced activity, while mutants at other nearby cysteine or histidine residues (Cys-1056 and His-1086) did not dramatically affect activity, and (ii) linker insertion mutants that altered the spacing between conserved cysteines (between Cys-228 and Cys-231 [10] and between Cys-1060 and Cys-1063) expressed partially active and inactive Ad Pol enzymes, respectively. Mutants at His-239 and Cys-241 abolished Ad-specific enzymatic activity and DNA binding in vitro and are predicted to lie within the loop of the hypothetical upstream zinc finger. Since most mutants in the downstream region were enzymatically inactive but could still bind NFI and pTP at nearly wt levels, it seems most likely that these alterations may have a direct effect on DNA binding, perhaps by disrupting a DNA-binding loop in a zinc finger. However, one cannot rule out the possibility that mutants in the downstream region have an indirect effect on activity, by disrupting some part of the Ad Pol structure without affecting the ability of the protein to bind to NFI and to pTP. Our current working hypothesis is that the downstream Cys-His region is responsible for the nonspecific interaction of Ad Pol with DNA during elongation or synthesis reactions (Fig. 7).

It is difficult to identify the exact nature of the defect in the upstream Cys-His region. Although mutants in this region appeared to retain Ad DNA elongation function, Ad-specific initiation function was lost. The loss of Ad-specific function suggested either that a binding site for a required protein factor had been abolished or that these mutants had lost the ability to bind Ad origin core sequences found between 9 and 18 nucleotides from each end of the Ad genome. These sequences have been shown to be essential for Ad DNA replication (8, 18, 19, 27, 41, 44, 50) and to be a site for weak binding of the pTP-Ad Pol heterodimer (43). NFI is thought to stabilize the binding of the pTP-Ad Pol heterodimer to this origin sequence (11, 30). That these mutants bound NFI and pTP at levels comparable to those of wt Ad Pol suggests that an interaction with DNA has been disrupted. Since DNA elongation is still functional in these mutants, it is possible that this upstream domain plays a role in recognition of the Ad-specific sequences in the core origin of replication. Although the structure of this upstream Cys-His-rich region is not known, it behaves like a zinc finger domain, and the role of these domains is DNA sequence recognition is well known (17, 22, 24, 33). Perhaps Ad Pol has two DNA binding domains: one encoded in the downstream Cys-His region that is responsible for binding to DNA primer-template complexes and another encoded in the upstream Cys-His region that participates in the recognition of Ad origin sequences (Fig. 7). This second Ad-specific recognition is probably very weak (43) and may require the protein-protein interaction with NFI to help bring Ad Pol to the correct position on the Ad origin to allow binding (11, 30). Since NFI is not required for Ad4 DNA replication, this Ad-specific origin interaction mediated by the Ad4 DNA Pol may be

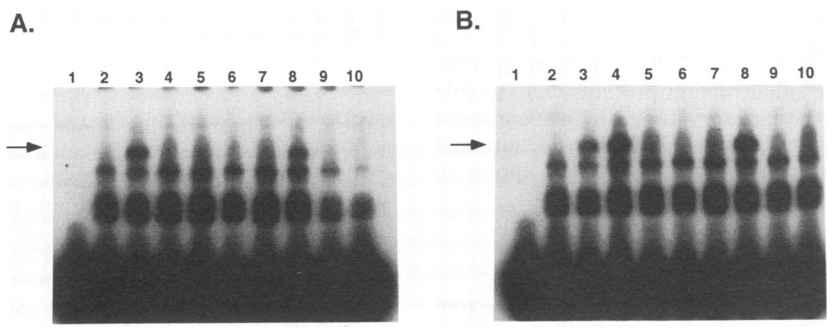

FIG. 6. Gel retardation analysis for the determination of the ability of Ad Pol mutants to bind to DNA, using an Ad origin sequence, TD16. Each mutant Ad Pol extract was reacted with end-labeled TD16 in the presence of pTP, and the mixture was analyzed by electrophoresis on a nondenaturing 5% polyacrylamide gel. The arrows indicate the specific shifted complex formed by binding Ad Pol and pTP to TD16. (A) Effects of mutations in the left Cys-His region on DNA binding. Lanes: 1, free oligonucleotide TD16; 2, negative control (vTF7-3); 3, wt; 4, C228S; 5, C231S; 6, H239S; 7, C241S; 8, H252S; 9, Ad Pol only; 10, pTP only. (B) Effects of mutations in the right site. Lanes: 1, free TD16; 2, negative control (vTF7-3); 3, wt; 4, C1056S; 5, *li*1061; 6, C1060S; 7, C1063S; 8, H1086S; 9, C1087S; 10, C1090S.

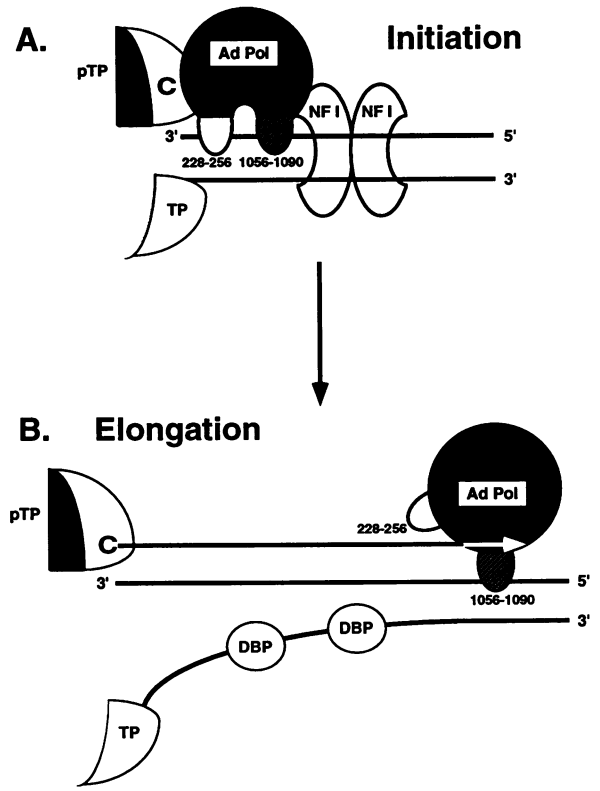

FIG. 7. Model for the interaction of Cys-His-rich domains of Ad Pol with Ad DNA. (A) During initiation, Ad Pol is associated with pTP and NFI; this complex then binds to the Ad DNA origin. During origin-specific DNA binding, both the upstream Cys-His-rich region (amino acids 228 to 256; shown as a white domain) and the downstream Cys-His-rich region (amino acids 1056 to 1090; shown as a hatched domain) recognize the origin sequences. dCTP (shown as C) is then covalently attached to the pTP molecule in the complex. NFI probably enhances the stability of this preinitiation complex (30). (B) During elongation, the upstream domain is no longer involved in DNA binding and the downstream Cys-His-rich region alone is sufficient for Ad Pol to interact with DNA as it moves down the DNA.

stronger; substitution of this region of Ad2 DNA Pol with the corresponding region of Ad4 DNA Pol might result in an enzyme that no longer requires NFI for in vitro Ad2 DNA replication.

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