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Biochemistry

Sequence and expression in *Escherichia coli* of the 40-kDa subunit of activator 1 (replication factor C) of HeLa cells

(DNA synthesis/DNA polymerases δ and ε/proliferating-cell nuclear antigen)

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ABSTRACT **Activator 1 (A1; also called replication factor C), in conjunction with proliferating-cell nuclear antigen (PCNA), is essential for the elongation of primed DNA templates by DNA polymerases δ and ε. A1 contains five distinct subunits of 145, 40, 38, 37, and 36.5 kDa. Here we describe the isolation, sequence, and bacterial expression of a cDNA coding for the 40-kDa subunit. In keeping with the presence of an ATP-binding motif, the bacterially expressed 40-kDa subunit binds ATP. The interaction between the 40-kDa subunit and ATP was reduced by the addition of PCNA. In addition, antibodies raised against the 40-kDa subunit abolished the A1- and PCNA-dependent synthesis of DNA catalyzed by polymerase δ. The putative amino acid sequence of the 40-kDa subunit of A1 revealed significant homology with the bacteriophage T4 gene *44* protein and, to a lesser degree, with the τ and γ subunits of *Escherichia coli* DNA polymerase III holoenzyme.**

Activator 1 (A1) is an auxiliary protein that, in conjunction with proliferating-cell nuclear antigen (PCNA), is required for the elongation of primed DNA templates by DNA polymerase (pol) δ and pol ε (1–6). Purified A1 from HeLa cells, which is identical to replication factor C (RF-C) contains five discrete subunits of 145, 40, 38, 37, and 36.5 kDa, all of which cosediment with A1 activity during glycerol gradient centrifugation (4–6). This multisubunit protein interacts with primed DNA to form a complex that in the presence of ATP binds PCNA. The complex of primed DNA·A1(ATP)·PCNA can then bind pol δ or pol ε to form a complex that elongates primer ends when supplemented with dNTPs (3, 7). Thus, A1 is important in the activation of low levels of primer ends, since pol δ (or pol ε), alone or when combined with PCNA, elongates such substrates poorly. A1 (RF-C) contains DNA-dependent ATPase activity that is stimulated by PCNA and the human single-stranded DNA-binding protein (HSSB), and the hydrolysis of ATP is essential for A1-dependent pol δ or pol ε activity (6, 8). Footprinting and UV crosslinking experiments demonstrated that RF-C binds to the primer–template junction through the 145-kDa subunit whereas the 40-kDa polypeptide binds ATP (9).

The action of A1 and PCNA resembles the role of the accessory proteins required for elongation of primed templates by *Escherichia coli* pol III [γδ complex and the β subunit (10, 11)] or phage T4 pol [T4 gene products (gp) 44/62 and 45] (12–14). These proteins are essential for the processive action of these DNA polymerases. The 44/62, γδ, and A1 proteins are all multisubunit and contain DNA-dependent ATPase activity (15, 16). ATP is required for the interaction of T4 gp 45, the β subunit, and PCNA with their corresponding auxiliary proteins. In addition, the ATPase activity of T4 gp 44/62 is markedly stimulated by T4 gp 45, which is analogous to the activation of the ATPase activity of A1 by PCNA (8, 17–19). Indeed, some primary amino acid sequence similarities between human PCNA and T4 gp 45 have been suggested (8). Successful substitution of the phage T4 pol and *E. coli* pol III holoenzymes for the pol δ holoenzyme (pol δ/PCNA/A1 complex) during simian virus 40 replication *in vitro* has further demonstrated conservation of the elongation of DNA templates in both prokaryotic and eukaryotic systems (20).

We have isolated cDNAs coding for three of the five distinct subunits and determined their nucleotide sequences. Here we present the sequence of the full-length cDNA encoding the 40-kDa subunit of A1.* The predicted amino acid sequence of this protein showed significant homology with phage T4 gp 44 and, to a lesser extent, with the τ and γ subunits of *E. coli* pol III.

MATERIALS AND METHODS

Isolation and Sequence Analysis of the 40-kDa Subunit of A1. A1 was isolated from HeLa cells (6). To obtain peptide sequence of the separated subunits, 200 pmol (50 μg) of A1 was reduced in 0.1 M Tris·HCl, pH 8.0/1% SDS/0.02 M dithiothreitol for 60 min at 60°C. Cysteine residues were then carbamoylmethylated with 0.44 mM iodoacetamide at 22°C followed by incubation in the dark for 30 min. After SDS/PAGE, the separated proteins were electroblotted onto nitrocellulose (Bio-Rad) with 20% methanol/192 mM glycine/25 mM Tris·HCl, pH 8.3/0.05% SDS as the transfer buffer. The nitrocellulose filter was stained with Ponceaus S and regions containing each separated subunit were excised and individually blocked with polyvinylpyrrolidine 40. The bound proteins were digested on the matrix with trypsin in 0.1 M NH_4HCO_3, pH 7.9/5% acetonitrile overnight at 37°C, with a trypsin/substrate ratio of 1:10 (wt/wt) (21). Peptides were separated by HPLC on a Brownlee C_4 column (30 × 2.1 mm) with a trifluoroacetic acid/acetonitrile system. Peptides were eluted with a linear gradient of 0–70% acetonitrile, and isolated peptides were sequenced with an Applied Biosystems 477A sequenator.

Oligonucleotide Synthesis and cDNA Library Screening. A 51-nucleotide oligomer was synthesized based on a peptide sequence derived from the trypsin-digested 40-kDa subunit of A1. This probe was used to screen a λ ZAPII library containing HeLa cDNA inserts (Stratagene). Phage were plated on *E. coli* XL1-Blue and grown for 8 hr on NZY plates. Nitrocellulose filter (Hybond C-Extra, Amersham) replicas from plates were treated according to a standard protocol, prehybridized in 6× standard saline citrate (SSC)/5× Den-

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Abbreviations: pol, DNA polymerase; gp, gene product; PCNA, proliferating-cell nuclear antigen; ORF, open reading frame; A1, activator 1; RF-C, replication factor C; HSSB, human single-stranded DNA-binding protein; IPTG, isopropyl β-D-thiogalactopyranoside; BSA, bovine serum albumin.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M87338).

hardt's solution/50 mM sodium phosphate, pH 7/0.1% SDS/20% formamide with sheared salmon sperm DNA (Sigma) at 0.1 mg/ml for 6 hr at 42°C, and hybridized for 12–16 hr at 42°C in the same solution containing the 5′-^{32}P-labeled oligonucleotide. Filters were then washed four times at room temperature with 6× SSC/0.1% SDS, twice with 0.2× SSC/0.1% SDS for 20 min, and finally with 0.1× SSC/0.1% SDS at 37°C for 30 min. Positive plaques detected on autoradiographs were subjected to two more cycles of purification.

Subcloning and DNA Sequence Analysis. The cDNA insert in the λ phage was recovered by *in vivo* excision using the helper phage R408 (22). The double-stranded templates were sequenced by the dideoxy method using T7 pol. The cDNA was sequenced bidirectionally using synthetic oligonucleotide primers based on previously determined sequences.

Expression of the 40-kDa Subunit in *E. coli*. The bacteriophage T7-based expression system was used to overproduce the cloned subunit in *E. coli*. The 1.4-kilobase-pair *Eco*RI fragment containing the putative open reading frame (ORF) encoding the 40-kDa subunit was inserted into the *Eco*RI site of the expression plasmid pET-5a, placing the ORF under the control of the strong translation initiation signal of T7 gp 10. This construct was transformed into *E. coli* BL21(DE3) cells, which harbor a lysogen containing the T7 pol gene under the control of the isopropyl β-D-thiogalactopyranoside (IPTG)-inducible *lac* UV5 promoter.

For induction of the recombinant protein, freshly transformed bacteria were grown overnight in Luria broth containing 0.4% glucose and 0.5 mg of ampicillin per ml at 37°C. The overnight culture was diluted 1:100 in the above medium and grown at 37°C for 2–3 hr, to an OD_{600} of 0.6. IPTG (0.4 mM) was added and the mixture was incubated for an additional 3 hr. The bacterial cells were pelleted, resuspended in sample buffer, and then subjected to SDS/10% PAGE.

The expressed protein was insoluble. However, extraction of the insoluble 40-kDa protein with 6 M urea, followed by chromatographic procedures, solubilized the protein, as will be described elsewhere. Antibodies against the 40-kDa subunit were prepared commercially by the Pocono Rabbit Farm (Canadensis, PA).

Immunoblotting. Proteins were transferred electrophoretically from SDS/polyacrylamide gels to nitrocellulose membranes in 25 mM Tris·HCl, pH 8.3/192 mM glycine/20% methanol. After transfer, filters were blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline (25 mM Tris·HCl, pH 7.5/150 mM NaCl) for 1 hr at room temperature and then incubated with the same solution containing polyclonal antibodies (diluted 1:2000) directed against the purified A1 multisubunit complex for 2 hr at room temperature. The filters were then washed four times with Tris-buffered saline/0.05% Tween-20, incubated with an anti-mouse alkaline phosphatase conjugate (diluted 1:2000), washed again, and subjected to color development with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrate.

Antibody Inhibition Assays. A1 purified from HeLa cells was preincubated on ice for 30 min in 20 μl of 40 mM Tris·HCl, pH 7.8/7 mM $MgCl_2$/0.5 mM dithiothreitol containing BSA (167 μg/ml) and either preimmune serum or polyclonal antiserum directed against the 40-kDa subunit of A1. The other components required for the assay of A1, including 0.1 μg of $(dA)_{4000}$·$(dT)_{12-18}$ at a nucleotide ratio of 20:1, 1 mM [^{3}H]dTTP, 2 mM ATP, pol δ (0.2 unit), HSSB (0.5 μg), and PCNA (100 ng), were then added and the mixture was incubated for 60 min at 37°C. The amount of dTMP incorporated into acid-insoluble material was determined.

Photocrosslinking. A reaction mixture (20 μl) containing 0.1–0.2 μg of the purified 40-kDa subunit from *E. coli*, 50 mM Tris·HCl (pH 7.5), 6 mM $MgCl_2$, 10% (vol/vol) glycerol, 10% dextrose, 5 mM dithiothreitol, 250 μg of BSA per ml, and 1.25 μM [α-^{32}P]ATP (400 Ci/mmol; 1 Ci = 37 GBq) was incubated at room temperature for 5 min, placed on ice, irradiated for 30 min under a germicidal UV lamp, and mixed with 20 μl of sample buffer for SDS/PAGE.

RESULTS

Isolation and Characterization of cDNA Clones. An 18-amino acid sequence was determined from a tryptic peptide of the 40-kDa subunit. This sequence was Ala-Leu-Asn-Asn-Leu-Gln-Ser-Thr-Phe-Ser-Gly-Phe-Leu-Phe-Ile-Asn-Ser-Glu and is underlined in Fig. 1. On the basis of this sequence and the eukaryotic codon usage (23), a 51-mer oligonucleotide was synthesized corresponding to amino

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CAATTTGAGTTTCCATTTCTCGGATTTGGGAACTGGTATAAGCATTGTCTGTGATGTAAA  60
CAAAGTCTTCAATATTTGGAGAAAACATCTCCTCATACTTGAGAGCACAAGAGGAAGAGA 120
GAGACCCTCACTGCTGGGGAGTCCCTGCCACACTCAGTCCCCCACCACACTGAATCGGAA 180
TTCCGAGAGGGAAGAGGAGGCGCGAGAATGGAGGTGGAGGCCGTCTGTGGTGGCGCGGGC 240
                        M  E  V  E  A  V  C  G  G  A  G
GAGGTGGAGGCCCAGGACTCTGACCCTGCCCCTGCCTTCAGCAAGGCCCCCGGCAGCGCC 300
E  V  E  A  Q  D  S  D  P  A  P  A  F  S  K  A  P  G  S  A
GGCCACTACGAACTGCCGTGGGTTGAAAAATATAGGCCAGTAAAGCTGAATGAAATTGTC 360
G  H  Y  E  L  P  W  V  E  K  Y  R  P  V  K  L  N  E  I  V
GGGAATGAAGACACCATGAGCAGGCTAGAGGTCTTTGCAAGGGAAGGAAATGTGCCCAAC 420
G  N  E  D  T  M  S  R  L  E  V  F  A  R  E  G  N  V  P  N
ATCATCATTGCGGGCCCTCCAGGAACCGGCAAGACCACAAGCATTCTGTGCTTGGCCCGG 480
I  I  I  A  G  P  P  G  T  G  K  T  T  S  I  L  C  L  A  R
GCCCTGCTGGGAGCACTCAAAGATGCCATGTTGGAACTCAATGCTTCAAATGACAGGGGC 540
A  L  L  G  A  L  K  D  A  M  L  E  L  N  A  S  N  D  R  G
ATTGACGTTGTGAGGAATAAAATTAAAATGTTTGCTCAACAAAAAGTCACTCTTCCCAAA 600
I  D  V  V  R  N  K  I  K  M  F  A  Q  Q  K  V  T  L  P  K
GGCCGACATAAGATCATCATTCTGGATGAAGCAGACAGCATGACCGACGGAGCCCAGCAA 660
G  R  H  K  I  I  I  L  D  E  A  D  S  M  T  D  G  A  Q  Q
GCCTTGAGGAGAACCATGGAAATCTACTCTAAAACCACTCGCTTCGCCCTTGCTTGTAAT 720
A  L  R  R  T  M  E  I  Y  S  K  T  T  R  F  A  L  A  C  N
GCTTCGGATAAGATCATCGAGCCCATTCAGTCCCGCTGTGCAGTCCTCCGGTACACAAAG 780
A  S  D  K  I  I  E  P  I  Q  S  R  C  A  V  L  R  Y  T  K
CTGACCGACGCCCAGATCCTCACCAGGCTGATGAATGTTATCGAGAAGGAGAGGGTACCC 840
L  T  D  A  Q  I  L  T  R  L  M  N  V  I  E  K  E  R  V  P
TACACTGATGACGGCCTAGAAGCCATCATCTTCACGGCCCAGGGAGACATGAGGCAGGCG 900
Y  T  D  D  G  L  E  A  I  I  F  T  A  Q  G  D  M  R  Q  A
CTGAACAACCTGCAGTCCACCTTCTCAGGATTTGGCTTCATTAACAGTGAGAACGTGTTC 960
L  N  N  L  Q  S  T  F  S  G  F  G  F  I  N  S  E  N  V  F

AAGGTCTGTGACGAGCCCCACCCACTGCTGGTAAAGGAGATGATCCAGCACTGTGTGAAT 1020
K  V  C  D  E  P  H  P  L  L  V  K  E  M  I  Q  H  C  V  N
GCCAACATTGACGAAGCCTACAAGATTCTTGCTCACTTGTGGCATCTGGGCTACTCACCA 1080
A  N  I  D  E  A  Y  K  I  L  A  H  L  W  H  L  G  Y  S  P
GAAGATATCATTGGCAACATCTTTCGAGTGTGTAAAACTTTCCAAATGGCAGAATACCTG 1140
E  D  I  I  G  N  I  F  R  V  C  K  T  F  Q  M  A  E  Y  L
AAACTGGAGTTTATCAAGGAAATTGGATACACTCACATGAAAATAGCGGAAGGAGTGAAC 1200
K  L  E  F  I  K  E  I  G  Y  T  H  M  K  I  A  E  G  V  N
TCTCTTTTGCAGATGGCAGGCCTCCTGGCAAGGCTGTGTCAGAAGACAATGGCCCCGGTG 1260
S  L  L  Q  M  A  G  L  L  A  R  L  C  Q  K  T  M  A  P  V
GCCAGTTAGAGCAGAGACTTCACTGACTGACTTACAGGTGCCCTATTCTGAGGTACAGGA 1320
A  S  *
GCCGCGGCTTCTGATGGGGGAAAATGCGCCTTAGGCTGAGCCAACATGACTGTCCCCCAA 1380
ACTCCAGTGGCTGGCCAGGCGCGGTAGTCACGCCTGTAATCCCAACACTTTGGGAGGCCG 1440
AGGCAGGTGGATCACCTGAGGTCAGAAGTTCAAGACCAGCCTGGCCAACATGGGGAAACC 1500
CTGTCTTTACTAAAAATATAAAAATTAGCTGGGTGTGGTGGCGGGCACCTGTAATCCCAG 1560
CTACTCGGGAGGCTGTGGCAGGCGAAATCGCTTGAACCCAGGAGGAGGAGGTGGAGGTTG 1620
CAGTGAGCCAAGATCACACCATTGCACTCCAGCCTGGGCGACAGAGACTCCATCTGGGGA 1680
AAAAAATTAAATAAATAAACTCCCGTGAC
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FIG. 1. cDNA sequence and deduced amino acid sequence of the 40-kDa subunit of A1. Nucleotides are numbered 5′ to 3′, beginning and ending with the *Eco*RI sites used to insert the cDNA into the λ ZAPII phage library. The deduced amino acid sequence is shown immediately below the corresponding nucleotide sequence. Underlined amino acid sequence was obtained from sequencing of tryptic peptides derived from SDS/PAGE-purified 40-kDa subunit; the single discrepancy between the peptide sequence and the predicted sequence is described in the text.

acids 2–18 (5′-CTGAACAACCTGCAGTCCACCTTCTC-TGGCTTCCTGTTCATCAACTCTGAG-3′). This labeled oligonucleotide was used to probe a HeLa cell cDNA library in λ ZAPII. Screening of 5×10^5 plaques yielded 4 positive plaques, which were further purified. The cDNA inserts from these clones were recovered from the λ ZAPII phage pBluescript plasmid by *in vivo* excision (22). The plasmid DNAs were purified and subjected to further restriction endonuclease mapping and nucleotide sequence analyses. These clones were related but were not full length, because they lacked the 5′ part of the ORF. To obtain clones that contained this sequence, the extreme 5′ oligonucleotide probe generated during sequencing was used to rescreen the same library. A single positive clone with a longer insert was isolated and characterized further.

Both strands of this clone were sequenced completely using synthetic oligonucleotide primers deduced from the sequence. This procedure revealed a 1709-bp insert between *Eco*RI sites. The nucleotide sequence of this insert (Fig. 1) contained an ORF that encoded 353 amino acid residues. The first initiation codon (ATG) was preceded by a 207-bp 5′ untranslated region, and the termination codon (TAG) was succeeded by a 440-bp 3′ untranslated region. Immediately upstream of the putative ATG initiation site, the sequence CGAGA (nucleotides 203–207) was detected that fulfills Kozak's criterion for a eukaryotic initiation site (24). A polyadenylylation signal, AATAAA, was observed at the end of the cDNA insert. The amino acid sequence obtained by directly sequencing tryptic peptides derived from the SDS/PAGE-purified 40-kDa subunit (underlined in Fig. 1) was detected. Residue 13, however, was determined to be leucine by the peptide sequencing rather than glycine as predicted from the cDNA sequence. In addition, the predicted molecular mass of the cDNA-encoded protein was 39 kDa, in keeping with the subunit molecular mass of the purified protein estimated by SDS/PAGE. For this reason, and because the bacterially overproduced protein can be recognized by antibodies against A1 (see below), we are confident that the isolated cDNA clone encodes the 40-kDa subunit of A1.

Homology Between the 40-kDa Subunit of A1, Phage T4 gp 44, and τ (or γ) Subunit of *E. coli* pol III Holoenzyme. The deduced amino acid sequence of the 40-kDa subunit of A1 was compared with that of a functional analogue, T4 gp 44 (25). The two proteins are 36% identical over a 150-amino acid overlap (Fig. 2*A*); when conservative amino acid substitutions are included, the sequence similarity is >68%. The homology between these two proteins is substantial throughout the coding region, although it is weakest at the N and C termini (data not shown). The 40-kDa subunit of A1 contains an adenine nucleotide-binding consensus sequence identical to that found in the T4 gp 44 protein (Fig. 2*B*). The 40-kDa subunit also shows limited regional similarity (20% identity and 70% conservation) to the accessory protein τ (or γ) of the *E. coli* pol III holoenzyme, which extends over a 220-amino acid overlap (data not shown). The consensus sequence for a putative ATP binding site is almost identical for these two proteins (Fig. 2*B*). Zinc finger and/or leucine zipper motifs, functional domains found in many DNA-binding proteins, were not detected in the 40-kDa coding region.

Expression of the 40-kDa Subunit in *E. coli*. The cDNA coding region for the 40-kDa subunit was cloned into the bacterial expression vector pET-5a. The ORF was placed under the control of the T7 RNA polymerase promoter. The plasmid is carried in *E. coli* strain BL21(DE3), which harbors a lysogen containing the T7 RNA polymerase gene whose expression is controlled by the *lac* UV5 promoter. The T7 expression vector contains the ATG initiation codon derived from the T7 gp 10 and has efficient translation initiation signals downstream from the phage promoter of this gene.

A

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          40         50         60         70         80         90
40k EKYRPVKLNEIVGNEDTMSRLEVFAREGNVPNIIIAGP-PGTGKTTSILCLARALLGALK
    .:::: ...: .  ..... .. ....:..:.:::. .: :::::::    .:.::  ...
T4gp44QKYRPSTIDECILPAEDKETFKSITSKGKIPHIILHSPSPGTGKTT----VAKALCHDVN
           20         30         40         50             60

      100        110        120        130        140        150
40k DAMLELNASNDRGIDVVRNKIKMFAQQKVTLPKGRHKIIILDEADSMT-DGAQQALRRTM
    ..: .. ...:  :: ::. .. :: .     .::.:.:..:: :. . ...:.  :..:
T4gp44ADM-NFVNGSDCKIDFVRGPLTNFASAASF--DGRQKVIVIDEFDRSGLAESQRHLRSEM
      70         80         90         100        110        120

      160        170        180        190
40k EIYSKTTRFALACNASDKIIEPIQSRCAVLRYTKLT
    : ::.. .. .. :. : ::.:.:::: :. ... :
T4gp44EAYSSNCSIIITANNIDGIIKPLQSRCRVITFGQPT
       130        140        150        160
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B

Protein	ATP binding motif	residues
40 kDa (AI)	Gly Pro Pro Gly Thr Gly Lys Thr Thr	76-84
T4gp44	Pro Ser Pro Gly Thr Gly Lys Thr Thr	50-58
γ subunit (PolIII)	Gly Thr Arg Gly Val Gly Lys Thr Ser	45-53

FIG. 2. (*A*) Homology between the 40-kDa subunit of A1 (40k) and T4 gp 44. Two dots between aligned amino acids indicate an exact match in the sequence; one dot denotes a conservative replacement. Dashes within the coding sequences indicate spaces inserted into one sequence by the ALIGN program to achieve optimal alignment of the two proteins. (*B*) Homology of the ATP-binding motifs of the 40-kDa, T4 gp 44, and γ subunits.

The ATG codon lies within an *Nde* I restriction site in the vector pET-5a and contains an *Eco*RI cloning site 30 bp downstream. When the 1.4-kbp *Eco*RI fragment containing the ORF of the 40-kDa subunit is inserted into the vector *Eco*RI site, the translation product of this construct should contain an N-terminal fusion of 25 amino acids added to the 378 amino acids of the 40-kDa subunit. In this case, 15 of these amino acids are derived from the expression vector (between the initiation codon and the cloning site) and 10 amino acids are derived from 5′ untranslated region of the 40-kDa cDNA.

Bacteria harboring either the vector pET-5a alone or the vector containing the cDNA insert were grown for 2 hr at 37°C after IPTG induction and then lysed. Immunoblotting with polyclonal antibodies directed against the purified A1 multisubunit complex detected primarily the 145-, 40-, 37-, and 36.5-kDa subunits of A1 but not the 38-kDa subunit (Fig. 3, lane 1). Several bands below the 145-kDa band were

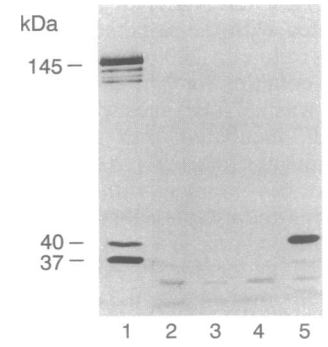

FIG. 3. Expression of the 40-kDa subunit of A1 in *E. coli*. An immunoblot of extracts (0.4 μg of protein in each lane) from bacteria transformed with the T7 phage expression vector pET-5a or the vector with the 40-kDa subunit cDNA was probed with polyclonal antibodies raised against the native A1 complex. Lane 1, 100 ng of A1 complex purified from HeLa cells; lanes 2 and 3, extracts from bacteria transformed with vector alone; lanes 4 and 5, extracts from bacteria transformed with the vector containing the cDNA insert. Extracts in lanes 3 and 5 were prepared from bacteria induced for 2 hr with IPTG; those in lanes 2 and 4 were prepared from uninduced cells.

observed, presumably representing proteolytic fragments derived from the 145-kDa subunit. A prominent 42-kDa band was observed in extracts of cells containing the cDNA insert after induction (Fig. 3, lane 5); this band was not observed either in uninduced cells containing the expression vector and insert (lane 4) or in cells containing the vector alone (lanes 2 and 3). The small increase in molecular mass of the 40-kDa subunit (lane 1) to 42 kDa after expression in *E. coli* (lane 5) was in good agreement with the fusion of an additional 25 codons onto the authentic ORF. In addition to the major 42-kDa protein, a small protein was detected that was not induced by IPTG. This protein may represent a bacterial protein that crossreacted with the primary or secondary antibodies. Approximately 5 μg of the 40-kDa subunit of A1 was produced from 10^9 cells. The fact that the cloned cDNA yielded a 40-kDa protein in *E. coli* that was recognized by antibodies directed against native A1 of HeLa cells further strengthened the identity of the isolated cDNA clone.

Polyclonal Antibodies Against the 40-kDa Subunit Specifically Inhibit A1-Dependent DNA Synthesis. Polyclonal antibodies preparations raised against the bacterially expressed protein specifically recognized the 40-kDa subunit of the purified A1 complex (data not shown). This antibody was tested for its ability to inhibit the A1-dependent synthesis of poly(dT) in the presence of poly(dA)·oligo(dT). For this purpose, purified A1 was first incubated with either preimmune or polyclonal serum, and then the components required for DNA synthesis were added. The polyclonal antibodies against the 40-kDa subunit strongly inhibited A1 activity in this assay. Preincubation of A1 with 0.5 μl of the polyclonal serum decreased nucleotide incorporation >80% compared with preincubation of A1 with the preimmune serum (Fig. 4*A*). The inhibition was completely reversed by the addition of 50 ng of purified A1 prior to the start of DNA synthesis (Fig. 4*B*). These results suggest that the inhibition of DNA synthesis by the polyclonal antibodies was due to the specific interaction between the 40-kDa subunits of A1 and the polyclonal antibody preparation.

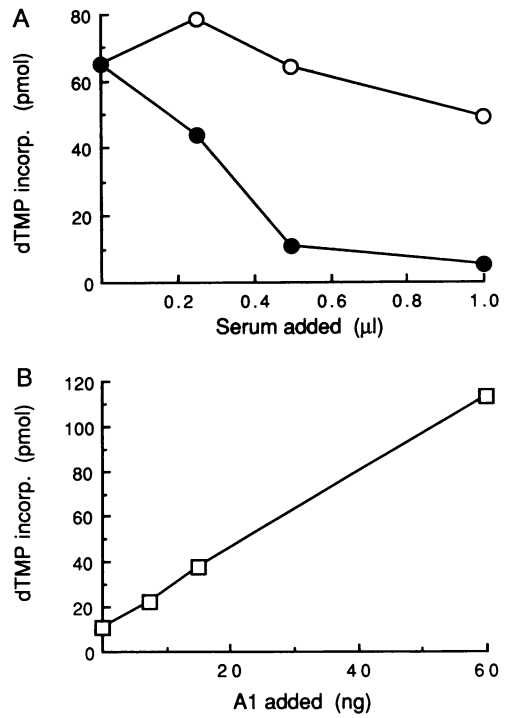

FIG. 4. (*A*) Influence of polyclonal antibodies directed against the 40-kDa subunit on DNA synthesis. Either preimmune serum (○) or polyclonal antiserum directed against 40-kDa subunit of A1 (●) was preincubated with 15 ng of A1 in 20 μl of 40 mM Tris·HCl, pH 7.8/7 mM $MgCl_2$/0.5 mM dithiothreitol containing BSA (167 μg/ml) for 30 min at 0°C. After addition of 0.5 μg of HSSB, 0.1 μg of PCNA, 0.2 unit of pol δ, 0.1 μg of $(dA)_{400}$·$(dT)_{12-18}$, and 1 mM [^{3}H]dTTP and incubation at 37°C for 60 min, the amount of acid-insoluble material was determined. (*B*) Effect of A1 on reactions inhibited with antibodies against the 40-kDa subunit. After incubation of A1 (15 ng) and immune serum (0.5 μl) on ice for 30 min, various amounts of A1 were added along with other components of the reaction. The amount of DNA synthesis after incubation for 60 min at 37°C is reported.

ATP Binding to the Bacterially Expressed 40-kDa Subunit. Since the 40-kDa subunit contains the consensus sequence for ATP binding (see Fig. 2*B*), the protein overproduced in *E. coli* was purified, solubilized, and used to determine its ATP-binding activity by UV cross-linking. After irradiation and SDS/PAGE, ATP complexed to the 40-kDa subunit as well as to a 36-kDa protein band (proteolyzed product of the 40-kDa subunit) (Fig. 5*A*, lane 2). The binding of ATP was abolished by the omission of Mg^{2+} (lane 1). The specificity of ATP binding to the 40-kDa subunit was tested by the addition of a 200-fold molar excess of each of the four unlabeled rNTPs. The binding of [α-^{32}P]ATP was eliminated by the addition of unlabeled ATP, whereas UTP, GTP, and CTP partially blocked the binding of labeled ATP (Fig. 5*A*, lanes 3–6). Previous studies indicated that A1 activity was maximally stimulated by ATP and poorly stimulated by the other rNTPs (6).

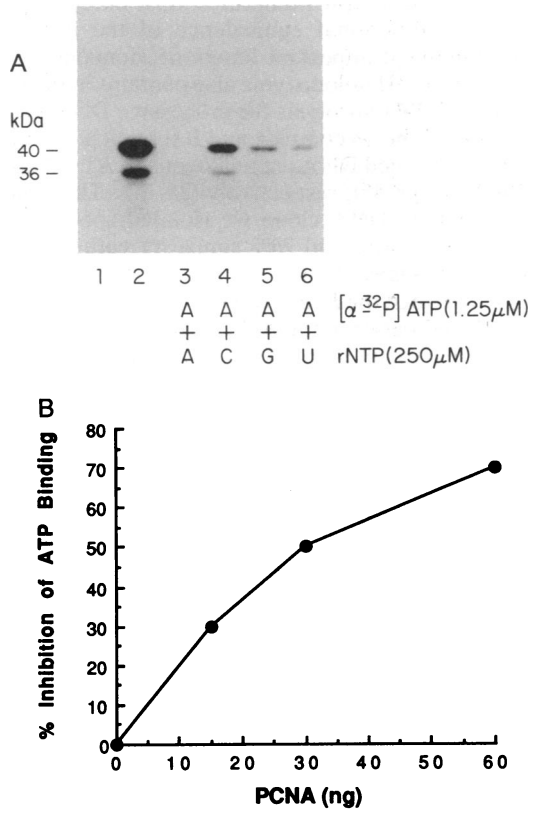

FIG. 5. (*A*) Photoaffinity labeling of the 40-kDa subunit by [α-^{32}P]ATP. Reaction mixtures (20 μl) containing 0.2 μg of the 40-kDa purified from *E. coli* and 1.25 μM [α-^{32}P]ATP were incubated at room temperature for 5 min. After irradiation, reactions were subjected to SDS/PAGE. Lane 1, $MgCl_2$ was omitted; lane 2, complete system; lanes 3–6, indicated rNTPs (250 μM) were added before incubation at room temperature for 5 min. The amount of ATP crosslinked to the 40- and 36-kDa (proteolyzed product formed from the 40-kDa subunit) polypeptides was quantified by scintillation counting; a total of 50 fmol of ATP was detected. (*B*) Effect of PCNA on the binding of ATP to the 40-kDa subunit. Reactions were carried out in the presence of 1.25 μM [α-^{32}P]ATP, 0.1 μg of the 40-kDa subunit, and various amounts of PCNA, with incubation at room temperature for 5 min. Samples were processed as described in *A*.

ATP binding was also decreased by the addition of PCNA (Fig. 5*B*), suggesting an interaction between PCNA and the 40-kDa subunit of A1. In the absence of A1, no ATP was crosslinked to PCNA (data not shown).

DISCUSSION

We have isolated and sequenced a cDNA clone encoding the 40-kDa subunit of A1. The cloned cDNA contained a single ORF that encoded a protein of 39 kDa, consistent with the mass of the 40-kDa subunit of A1. The proof of the identity of the cDNA clone included (*i*) the match between the amino acid sequence of tryptic peptides derived from the 40-kDa protein with the predicted amino acid sequence of the cDNA clone, (*ii*) the expression of a 42-kDa protein in *E. coli* that was recognized by polyclonal antibodies directed against the multisubunit A1 complex, and (*iii*) the specific reaction of the 40-kDa subunit of A1 with polyclonal antibodies raised against the bacterially expressed fusion protein. For these reasons, we are confident that the isolated cDNA clone encodes the 40-kDa subunit of A1.

Biochemical analysis has demonstrated that A1 and PCNA are functional analogues of the phage T4 pol accessory proteins gp 44/62 and gp 45, respectively. The 40-kDa subunit of A1 shows 36% amino acid identity and 32% conservative substitutions when compared with the T4 gp 44 subunit. Such structural similarities most likely provide the basis for the functional equivalence of the proteins and probably represent important functional domains.

The *E. coli* pol III holoenzyme also contains many subunits and requires ATP hydrolysis for processive DNA synthesis. The function of the $\gamma\delta$ complex and β subunit and their order of addition to primed DNA are analogous to A1 (T4 gp 44/62) and PCNA (T4 gp 45), respectively (25, 26). The amino acid sequence of the cDNA clone we isolated shows some homology (20% identity and 70% similarity with conservative amino acid changes) to the *E. coli* τ or γ proteins. The homology between the *E. coli* auxiliary factors is less obvious than that found between the 40-kDa subunit and T4 gp 44. In addition, comparison of the deduced amino acid sequence of PCNA with the *E. coli* β subunit failed to reveal significant homology. However, elucidation of the three-dimensional structure of the β subunit has revealed that each monomer of β consists of three structurally identical domains. Analysis of the hydrophobic cores of each of these domains showed that human and yeast PCNAs are likely to have two structure modules per monomer, each of which has a conserved hydrophobic core similar to that of β. This strongly suggests that these proteins have similar three-dimensional structures (J. Kuriyan and M. O'Donnell, personal communication).

Since much of the overproduced 40-kDa subunit of A1 from *E. coli* was insoluble, we used a urea treatment to purify and solubilize the protein. The 40-kDa protein cloned in *E. coli* binds ATP, a finding consistent with the observation that the 40-kDa subunit of the RF-C complex was crosslinked to ATP. The observation that PCNA reduced the crosslinking of ATP to the 40-kDa subunit suggests that PCNA may interact with this subunit.

T4 gp 44 has been shown to bind ATP by UV crosslinking experiments. Direct enzymatic assay revealed that the T4 gp 44 subunit alone possesses DNA-dependent ATPase activity, as does the T4 gp 44/62 complex (27). It has been proposed that the T4 gp 62 subunit facilitates the interaction of T4 gp 44 and gp 45. The sequence homology between the 40-kDa A1 subunit and T4 gp 44 suggests that the 40-kDa A1 subunit should also possess DNA-dependent ATPase activity. However, we detected no ATPase activity with the bacterially expressed 40-kDa subunit despite its ATP-binding activity. This suggests that the ATPase activity may require the interaction of the 40-kDa subunit with other A1 subunits. It is also possible that 40-kDa protein isolated here was partially inactivated by the urea treatment used to solubilize the protein. Overexpression of this subunit in the baculovirus expression system may overcome this problem.

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