The Primary Structure of the 32-kDa Subunit of Human Replication Protein A*

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Replication protein A (RP-A) is a complex of three polypeptides of molecular mass 70, 32, and 14 kDa, which is absolutely required for simian virus 40 DNA replication in vitro. We have isolated a cDNA coding for the 32-kDa subunit of RP-A. An oligonucleotide probe was constructed based upon a tryptic peptide sequence derived from whole RP-A, and clones were isolated from a λgt11 library containing HeLa cDNA inserts. The amino acid sequence predicted from the cDNA contains the peptide sequence obtained from whole RP-A along with two sequences obtained from tryptic peptides derived from sodium dodecyl sulfate-polyacrylamide gel-purified 32-kDa subunit. The coding sequence predicts a protein of 29,228 daltons, in good agreement with the electrophoretically determined molecular mass of the 32 kDa subunit. No significant homology was found with any of the sequences in the GenBank data base. The protein predicted from the cDNA has an N-terminal region rich in glycine and serine along with two acidic and two basic segments. Monoclonal antibodies have been raised against the 32-kDa subunit of RP-A. This monoclonal antibody against the 32-kDa subunit inhibits DNA replication in vitro.

Efforts to understand the replication of chromosomal DNA in animal cells have long been frustrated by the large size and complexity of their genomes. In order to overcome this problem, many investigators have used viral genomes as model systems for studying the processes involved in DNA replication. Simian virus 40 (SV40) has been highly purified: DNA polymerase α-primase complex (Wold et al., 1989); topoisomerases I and II (Yang et al., 1987); proliferating cell nuclear antigen (Prellic et al., 1987; Wold et al., 1988, 1989); replication protein C (the catalytic subunit of protein phosphatase 2A) (Virshup and Kelly, 1988); and replication protein A (RP-A) (Wold and Kelly, 1988; Virshup et al., 1989), also known as replication factor A (Fairman and Stillman, 1988) or HeLa SSB (Ishimi et al., 1988). RP-A is absolutely required for DNA replication in a reconstructed system (Wold and Kelly, 1988; Fairman and Stillman, 1988; Ishimi et al., 1988). It is believed that RP-A participate in a very early step in initiation because it is required for T antigen-dependent origin-dependent unwinding of the DNA template (Wold and Kelly, 1988). It is not known whether RP-A participates in the elongation step of replication as well as in initiation. RP-A is purified from HeLa cells as a complex consisting of three subunits of 70, 32, and 14 kDa. SV40 DNA replication requires only a single virus-encoded protein, large T antigen, the other replication functions being provided by the host cell.

In order to identify the cellular proteins involved in SV40 DNA replication, our laboratory has used a hypotonic extract of human tissue culture cells in which SV40 DNA is able to replicate in vitro (Li and Kelly, 1984, 1985). DNA replication in vitro shares many characteristics with SV40 DNA replication in vivo, including a requirement for the SV40 origin of replication, a requirement for large T antigen, and a requirement that extracts be made from cells that are permissive for viral replication in vivo (Li and Kelly, 1984, 1985; Stillman and Gluzman, 1985; Wobbe et al., 1985). Our laboratory has undertaken fractionation of crude extracts made from HeLa cells and reconstitution of replication activity. Through this approach, we have obtained evidence for the involvement of at least seven cellular factors in the complete replication of SV40 origin-containing DNA (Wold et al., 1989). Five of these have been highly purified: DNA polymerase α-primase complex (Wobbe et al., 1987; Wold et al., 1989); topoisomerases I and II (Yang et al., 1987); proliferating cell nuclear antigen (Prellic et al., 1987; Wold et al., 1988, 1989); replication protein C (the catalytic subunit of protein phosphatase 2A) (Virshup and Kelly, 1988); and replication protein A (RP-A) (Wold and Kelly, 1988; Virshup et al., 1989), also known as replication factor A (Fairman and Stillman, 1988) or HeLa SSB (Ishimi et al., 1988). RP-A is absolutely required for DNA replication in a reconstructed system (Wold and Kelly, 1988; Fairman and Stillman, 1988; Ishimi et al., 1988). It is believed that RP-A participate in a very early step in initiation because it is required for T antigen-dependent origin-dependent unwinding of the DNA template (Wold and Kelly, 1988). It is not known whether RP-A participates in the elongation step of replication as well as in initiation. RP-A is purified from HeLa cells as a complex consisting of three subunits of 70, 32, and 14 kDa (Wold and Kelly, 1988; Fairman and Stillman, 1988; Ishimi et al., 1988). This complex is very tightly associated since all three subunits cosediment in glycerol gradients run in 6 M urea (Fairman and Stillman, 1988) or in 1.7 M urea with 0.5 M KCl (Wold and Kelly, 1988). RP-A is an SSB, demonstrating a 1000-fold greater affinity for single-stranded DNA than for double-stranded DNA as measured by a nitrocellulose filter binding assay in which the binding of radiolabeled denatured DNA was competed with unlabeled single- and double-stranded DNA (Wold et al., 1989). Other investigators have reported that the affinity of RP-A for single-stranded DNA is 30-fold greater than for double-stranded DNA, as determined by measuring the DNA concentration at which 50% of the DNA
is bound to a nitrocellulose filter (Fairman and Stillman, 1988). When the subunits of RP-A were separated on an SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with radiolabeled single-stranded DNA, only the 70-kDa subunit was detected (Wold et al., 1989), indicating that the single-stranded DNA-binding activity may reside exclusively in this subunit. Other SSDs, including E. coli colI SSb and the adenovirus DNA-binding protein, are able to substitute for RP-A in the initial unwinding step in SV40 DNA replication, but they are unable to substitute for RP-A in the complete DNA replication reaction (Wold et al., 1987; Dean et al., 1987; Virshup and Kelly, 1989). This suggests either that a specific replication complex is formed with RP-A which cannot form with other SSBs or that RP-A has some additional essential activity beyond single-treatment DNA binding. RP-A was assayed for various enzymatic activities including ATPase, GTPase, 3′→5′ exonuclease, endonuclease, helicase, and topoisomerase I; none of these activities was found in RP-A (Wold et al., 1989).

In order to understand better the role of RP-A in DNA replication as well as the nature of the strong interactions among the three subunits, we have undertaken the isolation and sequencing of the cDNAs coding for the three polypeptide subunits. We report here the cloning of a cDNA coding for the 32-kDa subunit.

**Materials and Methods**

**Purification of RP-A**—RP-A was purified as described (Wold and Kelly, 1988) except that the 1.3 M KSCN wash from the Affi-Gel Blue column was concentrated and desalted by passing it over a small (0.2 ml) hydroxylapatite column and eluting bound material with buffer containing 50 mM potassium phosphate (Wold and Kelly, 1988). Peak fractions had a protein concentration as high as 1 mg/ml.

**Peptide Sequencing**—Peptide sequence was obtained from both whole RP-A and from the separated subunits. Approximately 1 nmol (150 μg) of RP-A was reduced in 100 mM Tris (pH 8.0), 1% SDS, 20 mM dithiothreitol for 60 min at 60 °C. After cooling to room temperature, cysteine residues were carboxymethylated by the addition of 0.05 volume of 0.44 M iodoacetamide (freshly made) with incubation at room temperature in the dark for 30 min. Then 9 volumes of 20 °C ethanol were added along with 3.8 μg (2.5% w/w relative to RP-A) of 1.1-oxalamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma; prepared from bovine pancreas). The protein was allowed to precipitate overnight at -20 °C and then was collected by centrifugation and resuspended in 0.3 ml of 100 mM NH4HCO3 (pH 7.5). An additional 3.85 μg of trypsin was added to make a final ratio of 1 μg of trypsin/20 μg of RP-A, and incubation was carried out for 4 h at 37 °C. Samples were frozen in liquid N2 and stored at -70 °C.

For sequencing the separated subunits, 150 μg was reduced, carboxymethylated, and ethanol precipitated as above, without trypsin, and then was subjected to SDS-polyacrylamide gel electrophoresis in a Mini Protean II (Hoefer Scientific) using a 10% running gel and a 5% stacking gel (Laemmli, 1970). The protein was then transferred to nitrocellulose filters that were prehybridized in 0 × SSPE, 5 × Denhardt’s solution, 0.1 mg/ml sheared salmon testes DNA (Sigma), 0.1% SDS, and 20% formamide for 2–8 h at 37 °C. The filters were then hybridized with the overnight at 37 °C. The filters that had been 5′ end labeled with [γ-32P]ATP using T4 polynucleotide kinase (U. S. Biochemicals). Filters were then washed twice, with brief shaking, at room temperature in 6 × SSC, 0.1% SDS, shaken in 6 × SSC, 1% SDS at room temperature for 30 min, and then washed once in 0.2 × SSC, 0.1% SDS for 30 min at 42 °C. All positive plaques were purified through two additional rounds.

**Subcloning and DNA Sequencing**—Human cDNA inserts were excised from Agt11 by cutting with EcoRI and cloned into the EcoRI site of the polylinker of the plasmid pBlueScript KS+ (Strategene, subsequently designated pBS). DNA sequencing was performed by a method described by Hunkapiller and Clogston (1987) (U. S. Biochemicals) according to the manufacturer’s instructions. Sequencing was done directly on double-stranded plasmids following denaturation by alkali and renaturation at 37 °C as described by Ausubel et al. (1987). Plasmids prepared by the boiling miniprep procedure (Holloway and Quigley, 1981) were extracted twice with phenol-chloroform, l:1, and chloroform, ethanol precipitated, digested with 1250 units of RNase T1 for 1 h at 37 °C, and extracted and precipitated again prior to sequencing. The ends of the cDNA insert were sequenced using the KS and SK 17-mer primers as well as the M13 forward and reverse 20-mer primers (Strategene).

Internal sequences of the cDNA were determined from deletions generated by digesting with restriction enzymes that cut within the cDNA and within the pBS polylinker. Some regions of the cDNA were sequenced by inserting Sau3Al subfragments into the BamHI site in the pBS polylinker. The recombinant plasmid containing the RP-A-32-kDa cDNA cloned in the EcoRI site of pBS will subsequently be referred to as pLE1. The entire cDNA was sequenced on both strands and most regions were read on at least two separate sequencing gels runs.

**Bacterial Expression of Cloned Protein**—The cloned gene was overexpressed in E. coli from the bacteriophage T7 10 promoter in a host in which T7 RNA polymerase is carried on a A-prophage and is expressed from the E. coli lacUVS promoter under the inducible control of the lac repressor. The plasmid was a gift of Dr. F. W. Studier, Brookhaven National Laboratory), which contains the T7 promoter, was cut at the single NotI site. The 5′ overhang was filled in with Klenow polymerase, and an 8-nucleotide-long BamHI linker (New England BioLabs) was ligated onto the ends. The RP-A-32-kDa cDNA insert was excised from pBS. First, the pLE1 coexpressed plasmid was cut in the polylinker with EcoRI. Second, a BamHI 8-nucleotide linker was ligated onto the EcoRI end. Third, both pET-8c and pLE1 were cut with BamHI, and the 4.5-kilobase linearized pET-8c as well as the 1.7-kilobase cDNA fragment from pLE1 were gel purified. These two fragments were ligated together and transformed into E. coli HN5174. Ampicillin-resistant colonies were grown on LB plates with polypropylene petri dishes and incubated at 37 °C for 4 h. Colonies were selected, and miniplasmids were purified. DNA was digested with EcoRI and BamHI, blotted onto a nitrocellulose filter, and hybridized with [32P]DNA probe at a specific activity of 106 cpm/μg. Filters were washed twice, with 0.2 × SSC, 0.1% SDS at room temperature for 30 min, and then washed once in 0.2 × SSC, 0.1% SDS for 30 min at 42 °C. All positive plaques were purified through two additional rounds.

For induction of a recombinant protein, freshly transformed bacteria were grown overnight in LB medium containing 50 μg/ml ampicillin. The next day, the overnight culture was diluted 1:100 in M9 medium containing 50 μg/ml ampicillin and grown at 37 °C for approximately 4 h to an OD600 of 0.6. Isopropyl-β-D-thiogalactoside (IPTG) was added to 0.4 mM to induce the T7 polymerase, and incubation was continued. After 30 min, rifampicin was added to a final concentration of 0.10 μg/ml. Bacteria were harvested after induction with IPTG by a brief centrifugation and resuspended in 1/140 volume of 50 mM Tris, 1 mM EDTA (pH 8.0). Cells were frozen and thawed three times, sonicated to break up high molecular mass DNA, and then digested into SDS-polyacrylamide gel loading buffer.
(Laemmli, 1970). Samples were boiled for 3 min prior to loading on an SDS-polyacrylamide gel for analysis.

Preparation of Monoclonal Antibodies—Two 10-week-old BALB/c female mice (Jackson Laboratories) were each immunized with 20 pg of RP-A in Freund’s incomplete adjuvant. An additional injection of 10 pg of RP-A in phosphate-buffered saline was given 1 week after the first injection of 10 pg of RP-A in phosphate-buffered saline was administered. Fusions were performed 4 days after the final injection. The fusions were screened against RP-A both by enzyme-linked immunoassay and by immunoblotting, and positives were cloned twice by limiting dilution. The antibody 71 that recognized the 32-kDa subunit of RP-A was of the IgG2a type, and the hybridoma was designated J71.3.5.1.3.

Immunoblotting—Proteins were transferred electrophoretically from SDS-polyacrylamide gels to a nitrocellulose membrane in 0.5 x Laemmli gel buffer, without SDS, but containing 20% methanol. The filter was stained briefly with 0.1% Ponceau S in 3% trichloroacetic acid and then destained with several changes of water. A 1:50-dilution of monoclonal antibody 71 in Western blot buffer (0.15 M NaCl, 0.01 M NaPO4, pH 7.5, 1 mM EDTA, 0.2% Triton X-100, 1 mM NaN3) containing 4% bovine serum albumin was added, and incubation was carried out for 2 h at room temperature. The blot was washed four times with Western blot buffer without bovine serum albumin. Incubation was then performed for 1 h at room temperature with a second antibody, rabbit anti-mouse IgG (Pep-Freez, affinity purified), at a 1:10,000 dilution, in Western blot buffer with 4% bovine serum albumin. The filter was washed as before. Finally, the blot was incubated for 90 min at room temperature with **125**I-labeled protein A (ICN; >30Ci/µg) diluted 1:10,000-fold in Western blot buffer with 4% bovine serum albumin and washed as before.

Inhibition of Replication with Monoclonal Antibodies—Crude HeLa cytoplasmic extract (Li and Kelly, 1984) was incubated, either with buffer H (Wold and Kelly, 1988) containing 15 mM KCl or with monoclonal antibody in buffer H containing 15 mM KCl, for 15 min at 0 °C, 15 min at 32 °C, and then for 10 min at 0 °C. At this point, buffers, SV40 origin-containing DNA, [32P]dCTP, and T antigen were added to the final concentrations required for in vitro replication as described previously (Wold et al., 1989). Replication was allowed to proceed for 2 h at 37 °C, and samples were treated as described (Wold et al., 1989).

RESULTS

Cloning of a cDNA for the 32-kDa Subunit of RP-A—A 22-amino acid peptide sequence was obtained from a tryptic digest in solution of whole RP-A. That sequence was read as Ser/Gin-Ala-Val-Asp-Phe-Leu-Ser-Asn-Glu-Gly-Ala-Ile-Tyr-Ser-Thr-Val-Asp-Asp-His-Phe-Lys. Using the table of preferred codon choice for human coding sequences (Lathe, 1985), a single nondegenerate 48-residue-long oligonucleotide of sequence 5'-GCTGTGGACTTCCTGTCCAATGAGGGC-3' was synthesized based upon amino acids 2-17. This probe was used to screen a λgt11 HeLa cell cDNA library at low stringency (a final wash at 0.2 x SSC). Approximately 200,000 plaques were screened, and 21 plaques that hybridized with the probe were plaque purified through three cycles of screening with the oligonucleotide. DNA was prepared from each of these bacteria and digested with EcoRI. The HeLa cDNA inserts released by this digestion were subcloned into the EcoRI site of pBS for further analysis. In order to determine whether any of these cDNAs encoded the amino acid sequence that had been obtained by peptide sequencing, the inserts were subjected to DNA sequencing using primers homologous to the polylinker region of the pBS vector. One of the cDNA inserts was found to be homologous with the oligonucleotide probe at 37 out of 48 positions. Translation of the DNA sequence yielded the amino acid sequence Gin-Ala-Val-Asp-Phe-Leu-Ser-Asn-Glu-Gly-His-Ile-Tyr-Ser- Thr-Val-Asp-Asp-His-Phe-Lys. This is identical to the amino acid sequence obtained by direct peptide sequencing, except that residue 11 was erroneously determined to be alanine by pep-
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PICTURE

FIG. 1. The sequence of the RP-A 32-kDa subunit cDNA and the predicted amino acid sequence. Nucleotides are numbered in the 5' to 3' direction, beginning and ending with the EcoRI sites used to insert the cDNA in Xgtll. The deduced amino acid sequence. The two amino acid sequences in boldface were read from the vector, the BamH site into which was ligated the BamHI site of the pLE1, which contains the K-H32-kDa subunit cDNA cloned into the polyliner EcoRI site, to produce the expression plasmid pLE2. Due to the way in which pLE2 was constructed, the open reading frame, beginning from the PET-8c AUG codon, contains 105 nucleotides between the PET-8c AUG and the first AUG codon of the cDNA open reading frame. As a result, the translation product will consist of an N-terminal fusion of 35 amino acids onto the 270 amino acids of the RP-A 32 kDa subunit. Of these 35 additional amino acids, 10 are derived from the vector, the BamHI linker, and polylinker sequences, while 25 are coded for by the 5'-untranslated region of the 32-kDa subunit cDNA.

Bacteria containing either pLE2 or the vector PET-8c were induced with IPTG, grown for 2 h at 37°C, and lysed. The lysates were electrophoresed on SDS-polyacrylamide gels, and the separated proteins were transferred to a nitrocellulose membrane. The filter was probed with a monoclonal antibody, designated 71, which was prepared as described under "Materials and Methods." When human RP-A is electrophoresed by guest on March 22, 2020http://www.jbc.org/Downloaded from

fingers (Berg, 1986) and leucine zippers (Landshultz et al., 1988), functional domains found in many DNA-binding proteins, as well as for the adenosine nucleotide-binding consensus sequence (Walker et al., 1982). None of these was found. We also failed to note any internally repeated domain within this protein.

Expression of the 32-kDa Subunit of RP-A in Bacteria—The cDNA coding for the 32 kDa subunit of RP-A was cloned into pET-8c, a bacterial expression vector in which exogenous genes can be expressed from the bacteriophage T7 φ10 promoter. The plasmid is carried in E. coli BL21 (DE3), a protease-deficient strain in which the bacteriophage T7 RNA polymerase is carried on a λ-prophage under the control of the lac operon. The orientation of the cloning sites in the vector is such that the T7 Tσ transcription termination sequence lies immediately downstream of the inserted gene. Logarithmically growing bacteria are induced with IPTG, an inducer of the lac operon, resulting in production of the T7 RNA polymerase and transcription of the inserted gene. The T7 expression vector used carries the AUG initiator codon from the T7 gene 10 protein, along with its efficient translation initiation signals, downstream from the phage φ10 promoter. This AUG codon lies within a Ncpl recognition site within the vector pET-8c. This site was converted to a BamHI site into which was ligated the BamHI site of the pLE1, which contains the K-H32-kDa subunit cDNA cloned into the polylinker EcoRI site, to produce the expression plasmid pLE2. Due to the way in which pLE2 was constructed, the open reading frame, beginning from the PET-8c AUG codon, contains 105 nucleotides between the PET-8c AUG and the first AUG codon of the cDNA open reading frame. As a result, the translation product will consist of an N-terminal fusion of 35 amino acids onto the 270 amino acids of the RP-A 32 kDa subunit. Of these 35 additional amino acids, 10 are derived from the vector, the BamHI linker, and polylinker sequences, while 25 are coded for by the 5'-untranslated region of the 32-kDa subunit cDNA.

Bacteria containing either pLE2 or the vector PET-8c were induced with IPTG, grown for 2 h at 37°C, and lysed. The lysates were electrophoresed on SDS-polyacrylamide gels, and the separated proteins were transferred to a nitrocellulose membrane. The filter was probed with a monoclonal antibody, designated 71, which was prepared as described under "Materials and Methods." When human RP-A is electrophoresed under these conditions, the monoclonal antibody recognizes only the 32-kDa subunit (Fig. 2, lane 1). The principal new protein produced when bacteria pLE2 are induced is a 32-kDa protein that is recognized by monoclonal antibody 71 (Fig. 2, lane 2). We estimate that approximately 2 μg of recombinant protein is produced/ml of cells. This 32-kDa protein recognized by the anti-RP-A monoclonal antibody is produced only at very low levels in uninduced cells containing pLE2 (Fig. 2, lane 4) and is not seen in bacteria containing the vector pET-8c, whether or not they are induced (Fig. 2, lanes 3 and 5). In addition to the predominant 32-kDa product, a slightly smaller species that is also recognized by the monoclonal antibody is produced in considerably smaller amounts upon induction of bacteria containing pLE2. The fact that the cloned cDNA codes for the production of a 32-kDa protein that is recognized by a monoclonal antibody against the 32-kDa subunit of RP-A confirms the identity of the cDNA that we have cloned. It might have been expected—since the bacterial expression construct has an additional 35 amino acids of open reading frame which are not present in the human cDNA—that the bacterially expressed 32-kDa subunit would be somewhat larger than the protein isolated from human cells. It may be that the bacterially expressed protein has undergone some proteolysis in the bacteria or upon cell lysis. It is also possible that the mammalian protein may contain some post-translational modifications, not produced in bacteria, which cause it to run a few kilodaltons larger on SDS-polyacrylamide gels than would be predicted by the size of the cDNA open reading frame. It is apparent from Fig. 2
that several proteins present in uninduced bacteria are recognized to some extent by the anti-32-kDa subunit monoclonal antibody. These species are not detected when the Western blot is probed only with rabbit anti-mouse IgG and protein A without the monoclonal antibody (data not shown). The strongest of these bands runs with a mobility just slightly less than that of the 32-kDa subunit of RP-A; this species may be a bacterial protein that shares some immunological characteristics with the mammalian protein.

A Monoclonal Antibody against the 32-kDa Subunit of RP-A Inhibits DNA Replication in Vitro—In order to examine further the role of the 32-kDa subunit of RP-A in DNA replication in vitro, we examined whether monoclonal antibody against the 32-kDa subunit of RP-A would inhibit DNA replication in a cell-free system. A crude HeLa cytoplasmic extract was preincubated with either 2.8 or 5.6 μg of ammonium sulfate-concentrated supernatant from the hybridoma line producing monoclonal antibody 71, and the additional components required for in vitro replication were then added. Replication was allowed to proceed for 2 h at 37 °C. The products of replication were electrophoresed on an agarose gel and analyzed by autoradiography. The monoclonal antibody against the 32-kDa subunit of RP-A strongly inhibited DNA replication. Preincubation with 5.6 μg of anti-32-kDa subunit monoclonal antibody produced a 74% inhibition compared with preincubation with antibody buffer alone, whereas preincubation with 2.8 μg of antibody resulted in a 72% inhibition of DNA replication (Fig. 3). This inhibition could be completely overcome by the addition of 200 ng of purified RP-A prior to the start of the replication reaction, indicating that the inhibition is due to a specific interaction between the antibody and RP-A and not some nonspecific inhibitory component in the antibody preparation.

**DISCUSSION**

We report the cloning of a cDNA that encodes the 32-kDa subunit of human RP-A, a protein absolutely required for SV40 DNA replication in vitro. The sole long open reading frame within the cDNA would code for a protein of molecular mass 29,228 daltons, a number in good agreement with the molecular mass of the 32-kDa subunit as measured on SDS-polyacrylamide gels. Within the open reading frame are found two peptide sequences identical to those obtained from direct sequencing of the 32-kDa subunit purified by SDS-polyacrylamide gel electrophoresis. The cloned cDNA encodes the production in E. coli of a 32-kDa protein that is recognized by a monoclonal antibody specific for the 32-kDa subunit of RP-A. The predicted amino acid sequence exhibits no significant homologies with any of the proteins in the sequence banks.

One interesting feature of the derived amino acid sequence is the presence of two acidic regions: one of 29 amino acids with a net charge of −7, and one of 24 amino acids with a net charge of −4. Similar acidic stretches are found in several yeast transcriptional activator proteins, including GAL 4 (Laughon and Gesteland, 1984), GCN 4 (Hinnebusch, 1984), and PHO 4 (Legrain et al., 1986). For example, a 19-amino acid region of GCN 4, with a net charge of −5, appears to be sufficient to allow transcriptional activation (Hope and Struhl, 1986). Current models of transcriptional activation propose that the transcriptional activators interact with DNA through a DNA-binding domain and interact with other proteins through the acidic activator domain. It is possible that the acidic regions of the 32-kDa subunit of RP-A are involved in protein-protein interactions with the other subunits of RP-A and perhaps with other proteins involved in replication. Now that the cDNA for the 32-kDa subunit is in hand and has been expressed in bacteria, mutagenesis can be undertaken to define the sequences within this protein necessary for the intersubunit interactions as well as interactions with other replication proteins. We have obtained the peptide...
sequence from the gel-purified 70 and 14-kDa subunits and are currently engaged in isolating eDNAs encoding these two proteins.

The exact role of the 32- and 14-kDa subunits of RP-A in DNA replication in vitro is still unknown. We have presented evidence that a monoclonal antibody directed against the 32-kDa subunit is able to inhibit replication, indicating that it does play an essential role in replication. RP-A is required both for complete replication as well as in the presynthetic template-unwinding reaction carried out in the presence of origin-containing DNA and large T antigen. The RP-A complex has single-stranded DNA-binding activity that is intrinsic to the 70-kDa subunit (Wold et al., 1989). The single-stranded DNA-binding activity of the 70-kDa subunit seems to be sufficient for presynthetic template unwinding, since single-stranded binding proteins from autologous sources will substitute for RP-A in this reaction. The single-stranded DNA-binding activity, however, appears not to be sufficient for the complete replication reaction, since SSBs from autologous sources will not substitute for RP-A in replication. It is possible that the 32- and 14-kDa subunits provide some enzymatic activities that are necessary for replication but not for unwinding. Another possibility is that the proteins involved in SV40 DNA replication form a large multiprotein complex such as has been proposed to be involved in the replication of E. coli (Baker et al., 1986) and bacteriophage λ-DNA (Dodson et al., 1986). There might then be very specific interactions between RP-A and other replication proteins which are unable to be reproduced by heterologous SSBs. The 32- and 14-kDa subunits of RP-A might play an essential role in mediating these protein-protein interactions. The ability to overexpress these proteins and carry out mutagenesis should allow delineation of the precise nature of the interactions in which these molecules participate.

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