The EMBO Journal vol.12 no.12 pp.4555–4566, 1993

The role of the 70 kDa subunit of human DNA polymerase α in DNA replication

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Communicated by D.Nathans

DNA polymerase α is the only enzyme in eukaryotic cells capable of starting DNA chains *de novo* and is required for the initiation of SV40 DNA replication *in vitro*. We have cloned the 70 kDa subunit of human DNA polymerase α (hereafter referred to as the B subunit) and expressed it as a fusion protein in bacteria. The purified fusion protein forms a stable complex with SV40 T antigen, both in solution and when T antigen is bound to the SV40 origin of DNA replication. Analysis of mutant forms of the B subunit indicates that the N-terminal 240 amino acids are sufficient to mediate complex formation. The B subunit fusion protein promotes formation of a complex containing T antigen and the catalytic subunit (subunit A) of DNA polymerase α, suggesting that it serves to tether the two proteins. These physical interactions are functionally significant, since the ability of T antigen to stimulate the activity of the catalytic subunit of DNA polymerase α is highly dependent upon the B subunit. We suggest that the interactions mediated by the B subunit play an important role in SV40 DNA replication by promoting DNA chain initiation at the origin and/or facilitating the subsequent priming and synthesis of DNA chains on the lagging strand template. The protein may play similar roles in cellular DNA replication.

Key words: DNA polymerase α/DNA replication/SV40

Introduction

In eukaryotic cells DNA replication is mediated by a complex multi-enzyme system whose activity is tightly regulated during the cell cycle. Although there is still much to be learned about the biochemical mechanism of eukaryotic DNA replication, considerable insight has been gained from the study of simple viral systems such as SV40. With the exception of the virus encoded protein, T antigen, the replication of the SV40 genome is completely dependent on cellular enzymes. The development of a cell-free system for SV40 DNA replication (Li and Kelly, 1984) has led to the identification of seven cellular factors which together with T antigen are necessary and sufficient to reconstitute DNA replication *in vitro* (Challberg and Kelly, 1989; Stillman, 1989; Hurwitz *et al.*, 1990; Fanning and Knippers, 1992). In this paper we describe the properties of a cellular polypeptide that appears to play an important role in organizing the DNA replication machinery for the efficient initiation and elongation of nascent DNA chains.

Studies of DNA replication *in vitro* have demonstrated that three proteins, DNA polymerase α–primase complex (DNA pol α), replication protein A (RP-A) and T antigen, are required for the initiation of DNA synthesis on duplex DNA molecules containing the viral origin of replication (Lee *et al.*, 1989; Tsurimoto *et al.*, 1990; Weinberg *et al.*, 1990). The first step in the initiation reaction is the ATP-dependent assembly of T antigen into a double hexamer at the origin of replication (Mastrangelo *et al.*, 1989; Parsons *et al.*, 1991; Virshup *et al.*, 1992). In the presence of RP-A, T antigen opens the two DNA strands near the origin, creating a region of single-stranded DNA (Dean *et al.*, 1987a; Dodson *et al.*, 1987; Wold *et al.*, 1987). The resulting structure serves as a template for the DNA polymerase α–primase complex which catalyzes the priming and synthesis of the first DNA chains (Tseng and Ahlem, 1984; Murakami *et al.*, 1986; Ishimi *et al.*, 1988; Wold *et al.*, 1989; Tsurimoto *et al.*, 1990; Weinberg *et al.*, 1990). As the replication forks advance the DNA polymerase α–primase complex continues to prime and extend new DNA strands on the lagging strand template. Other DNA polymerase activities appear to catalyze the processive extension of the first DNA chains on the leading strand template (Lee *et al.*, 1989; Tsurimoto *et al.*, 1990; Weinberg *et al.*, 1990). The high efficiency of the initiation reaction depends upon specific protein–protein interactions among the participating factors. Significant interactions have been detected between T antigen and DNA polymerase α–primase complex (Smale and Tjian, 1986; Gannon and Lane, 1987, 1990; Dornreiter *et al.*, 1990, 1993), between T antigen and RP-A (Dornreiter *et al.*, 1992) and between the DNA polymerase α–primase complex and RP-A (Dornreiter *et al.*, 1992). The interaction between T antigen and the DNA polymerase α–primase complex is particularly important, since the physical coupling of these two components of the replication apparatus increases the efficiency with which new DNA chains are initiated (Collins and Kelly, 1991; Erdile *et al.*, 1991; Melendy and Stillman, 1993).

DNA pol α is a four subunit enzyme that exhibits both DNA polymerase and DNA primase activities (Lehman and Kaguni, 1989). The central importance of this enzyme lies in the fact that it contains the only activity in eukaryotic cells that is capable of starting DNA chains *de novo*. The basic subunit structure of DNA pol α has been conserved in all eukaryotes, and each subunit has been shown to be essential for viability in yeast (Lucchini *et al.*, 1987; Plevani *et al.*, 1987; Pizzagalli *et al.*, 1988; Lehman and Kaguni, 1989; Foiani *et al.*, 1989; Brooke *et al.*, 1991). The largest (180 kDa) polypeptide of human DNA pol α (subunit A), which contains the DNA polymerase catalytic activity, has recently been cloned and expressed in active form (Wong *et al.*, 1988; Copeland and Wang, 1991). A physical interaction

between this subunit and T antigen has been reported (Dornreiter *et al.*, 1990, 1993). The primase activity of the complex resides in the two smallest subunits of molecular weight 58 and 48 kDa (subunits C and D, respectively) (Kaguni *et al.*, 1983; Tseng and Ahlem, 1983). The role of the remaining subunit (subunit B) has remained a mystery in spite of extensive work on the biochemical properties of DNA pol α purified from many sources. It is possible that subunit B plays some role in regulating the activity of DNA pol α in the cell cycle since it is phosphorylated specifically in G_2/M phase and is a substrate for the cell cycle dependent $p34^{cdc2}$ protein kinase (Nasheuer *et al.*, 1991).

In this paper we report experiments aimed at defining the role of the B subunit in DNA replication. The human subunit B was cloned and expressed in bacteria as a fusion protein. The purified protein was shown to be capable of binding to the SV40 T antigen, as well as to the catalytic subunit A of human DNA polymerase α. Under the conditions of our experiments the B subunit greatly enhanced the formation of a complex containing T antigen and the catalytic subunit of DNA pol α, suggesting that it serves as a molecular tether in this interaction. The physical interactions detected in these experiments were shown to be functionally important since T antigen stimulated the activity of the catalytic subunit on model templates only in the presence of subunit B. In addition, a fragment of subunit B capable of binding to T antigen, but not to the catalytic subunit of DNA pol α, inhibited SV40 DNA replication in the complete cell-free system. Based on these experiments, we suggest that the function of the B subunit is to couple the DNA polymerase α–primase complex to the DNA unwinding engine, thereby increasing the efficiency of initiation of the first DNA chains at the origin and facilitating the subsequent priming and synthesis of DNA chains on the lagging strand template. By analogy to the SV40 system, subunit B may play a role in coupling the polymerase–primase complex to the cellular initiation/elongation machinery.

Results

Cloning and characterization of the B subunit of human DNA polymerase α

The B subunit of DNA pol α was cloned by PCR methods using information derived from peptide sequence. Murine DNA pol α core enzyme was purified to apparent homogeneity (Prussak and Tseng, 1987) and the A and B subunits were separated by SDS–PAGE. The amino acid sequences of three tryptic peptides of the B subunit were obtained, and degenerate oligonucleotides corresponding to two of the peptides were used in PCRs to amplify a specific

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CTTGGGCGCAGGTCGGAGCTGGGTGGGCCGGCTCCCCGGCCTGGCTTGGGCGACCATGTCCGCATCCGCCCAGCAGCTGGCGGAGGAGCTGCAGATCTTCGGCCTAGACTGCGAGGAGGC  120
                                                          M  S  A  S  A  Q  Q  L  A  E  E  L  Q  I  F  G  L  D  C  E  E  A   22

TCTAATTGAGAAATTGGTAGAGCTTTGTGTTCAGTATGGACAGAATGAGGAGGGAATGGTAGGCGAGCTTATAGCCTTCTGCACCAGCACACATAAAGTTGGCCTTACCTCAGAGATCCT  240
 L  I  E  K  L  V  E  L  C  V  Q  Y  G  Q  N  E  E  G  M  V  G  E  L  I  A  F  C  T  S  T  H  K  V  G  L  T  S  E  I  L   62

GAACTCTTTTGAGCATGAGTTTCTGAGCAAAAGATTATCGAAAGCCAGGCATAGTACCTGCAAGGACAGTGGCCATGCAGGAGCTAGAGACATTGTTTCCATTCAAGAGCTAATTGAAGT  360
  N  S  F  E  H  E  F  L  S  K  R  L  S  K  A  R  H  S  T  C  K  D  S  G  H  A  G  A  R  D  I  V  S  I  Q  E  L  I  E  V  102
                                                                                                                        A

GGAAGAAGAAGAGGAAATCCTCTTGAACTCTTACACCACACCTTCAAAGGGTTCTCAGAAGCGAGCTATCTCTACCCCAGAAACCCCCCTAACAAAAAGGAGTGTGTCAACTCGTAGCCC  480
 E  E  E  E  E  I  L  L  N  S  Y  T  T  P  S  K  G  S  Q  K  R  A  I  S  T  P  E  T  P  L  T  K  R  S  V  S  T  R  S  P  142
          T        S                          P  L        V  S                             A  A
CCATCAGCTACTCTCACCGTCAAGTTTCTCTCCAAGTGCTACTCCCTCCCAGAAATACAACTCACGAAGTAACCGAGGAGAAGTGGTTACCTCCTTCGGCTTAGCACAGGGAGTATCTTG  600
 H  Q  L  L  S  P  S  S  F  S  P  S  A  T  P  S  Q  K  Y  N  S  R  S  N  R  G  E  V  V  T  S  F  G  L  A  Q  G  V  S  W  182
 R                                                   T     T              T        S        L
GTCTGGGAGAGGAGGAGCTGGAAACATCAGCCTGAAGGTCTTGGGATGTCCAGAGGCACTAACTGGGAGCTACAAATCCATGTTTCAGAAGCTCCCAGACATTCGAGAAGTTCTGACCTG  720
 S  G  R  G  G  A  G  N  I  S  L  K  V  L  G  C  P  E  A  L  T  G  S  Y  K  S  M  F  Q  K  L  P  D  I  R  E  V  L  T  C  222
          S     S  V              V     D        P                 A           Q     M  G  V                    S
TAAGATAGAAGAACTTGGCAGCGAACTCAAGGAACATTACAAGATTGAAGCTTTCACTCCTTTGCTAGCCCCAGCACAGGAGCCTGTCACTCTGCTGGGCCAGATTGGCTGTGATAGCAA  840
 K  I  E  E  L  G  S  E  L  K  E  H  Y  K  I  E  A  F  T  P  L  L  A  P  A  Q  E  P  V  T  L  L  G  Q  I  G  C  D  S  N  262
                                   H                    V                    I
CGGGAAGCTGAACAACAAGTCAGTGATTCTCGAGGGAGACCGGGAACATTCCTCGGGTGCTCAAATTCCAGTGGATTTATCTGAGCTTAAGGAATATTCTCTGTTTCCTGGACAGGTTGT  960
 G  K  L  N  N  K  S  V  I  L  E  G  D  R  E  H  S  S  G  A  Q  I  P  V  D  L  S  E  L  K  E  Y  S  L  F  P  G  Q  V  V  302
          S                       Q        Y
AATTATGGAAGGAATCAACACCACTGGTAGGAAACTTGTTGCCACCAAACTCTACGAGGGTGTGCCACTTCCATTTTATCAGCCCACTGAAGAGGATGCAGACTTTGAGCAAAGCATGGT 1080
 I  M  E  G  I  N  T  T  G  R  K  L  V  A  T  K  L  Y  E  G  V  P  L  P  F  Y  Q  P  T  E  E  D  A  D  F  E  Q  S  M  V  342
          F
CCTGGTTGCCTGTGGACCATACACCACATCTGACAGCATCACGTATGACCCCCTGCTTGACCTGATTGCTGTCATCAACCATGACCGGCCAGATGTCTGCATCCTGTTTGGCCCTTTCCT 1200
 L  V  A  C  G  P  Y  T  T  S  D  S  I  T  Y  D  P  L  L  D  L  I  A  V  I  N  H  D  R  P  D  V  C  I  L  F  G  P  F  L  382

GGAGTCTAAGCATGAACAGGTGGAGAATTGTCTACTGACAAGTCCATTTGAAGACATTTTCAAGCAGTGTCTACGAACAATTATTGAAGGCACAAGAAGCTCCGGCTCCCACCTTGTCTT 1320
 E  S  K  H  E  Q  V  E  N  C  L  L  T  S  P  F  E  D  I  F  K  Q  C  L  R  T  I  I  E  G  T  R  S  S  G  S  H  L  V  F  422

TGTCCCGTCATTGAGAGATGTGCACCATGAGCCTGTGTACCCCCAGCCGCCTTTCAGCTACTCCGATCTGTCTCGAGAGGACAAAAAGCAAGTACAGTTTGTGTCCGAGCCCTGCAGCCT 1440
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CTCCATAAACGGAGTGATCTTCGGCTTGACATCCACAGATCTGCTTTTCCACCTGGGGGCCGAGGAGATCAGTAGTTCTTCCGGAACTTCAGACAGATTCAGCCGAATACTCAAGCACAT 1560
 S  I  N  G  V  I  F  G  L  T  S  T  D  L  L  F  H  L  G  A  E  E  I  S  S  S  S  G  T  S  D  R  F  S  R  I  L  K  H  I  502

CTTGACCCAGAGGAGCTACTACCCACTCTACCCGCCCCAAGAAGACATGGCCATTGACTATGAGTCGTTCTATGTTTACGCACAGCTGCCTGTCACCCCAGATGTCCTCATCATCCCGTC 1680
 L  T  Q  R  S  Y  Y  P  L  Y  P  P  Q  E  D  M  A  I  D  Y  E  S  F  Y  V  Y  A  Q  L  P  V  T  P  D  V  L  I  I  P  S  542

AGAGCTGAGGTACTTCGTGAAGGATGTCCTCGGCTGTGTCTGTGTGAACCCTGGGCGCCTTACCAAAGGGCAGGTGGGAGGCACCTTCGCCCGACTCTACCTTAGGAGGCCGGCAGCGGA 1800
 E  L  R  Y  F  V  K  D  V  L  G  C  V  C  V  N  P  G  R  L  T  K  G  Q  V  G  G  T  F  A  R  L  Y  L  R  R  P  A  A  D  582

CGGGGCAGAGAGGCAGAGCCCATGCATTGCTGTGCAGGTCGTCAGGATCTGAGGCTTCTGTCCTCTGCTGTTCTCTGCTGTGTGGGCCCTTAAAGTCTTAGCCAAGAGCCAAGACATAGC 1920
 G  A  E  R  Q  S  P  C  I  A  V  Q  V  V  R  I

CCTGTGACAAGGTGAACAGTTGGGTGGGAAAGGAGAGAGGAGCCAGCCAGGGAGGGGCAGCTGCAGTGACCAGGCCCAGCAGGAGGACTTGTGCAGCCGGGCCTGCCTGTGAGTGGTGCC 2040
TCTCCTGGAAGAAGCTCTTGCTTCTCAGTCCATGCTCCGTGTCCAGAAGTAAGCCAGCTGTGGATCCCGCCCACTCAGAAAAGGCGAGAAGGCTTTGTGATTTTCTACATGAATCAAACA 2160
CAGAAACA
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Fig. 1. Nucleotide sequence and predicted amino acid sequence of the B subunit of human DNA polymerase α. Indicated below the sequence are the amino acid differences between the human cDNA and a mouse cDNA fragment. The murine fragment was obtained from cDNA using a PCR protocol described in Materials and methods. Amino acid sequences that are underlined indicate the position of the peptides specifying the degenerate oligonucleotides used in the PCR. The peptides were originally derived from murine DNA pol α subunit B (Prussak and Tseng, 1987).

fragment of mouse cDNA. Sequence analysis revealed that this fragment also encoded the third peptide, confirming its identity as a segment of the B subunit cDNA (Figure 1). The murine cDNA fragment was used as a probe to isolate a human cDNA clone encoding a protein with a predicted mol. wt of 66 kDa. The deduced amino acid sequence of the human B subunit exhibited 83% identity with the recently published sequence of the murine B subunit (Miyazawa *et al.*, 1993) and ~27% identity with the sequences of the B subunits of *Drosophila melanogaster* (Cotterill *et al.*, 1992) and *Saccharomyces cerevisiae* DNA pol α (D.Hinkle, personal communication). Dot matrix comparisons of the amino acid sequences of human, *Drosophila* and yeast B subunits revealed that regions of identity were largely in the C-terminal two-thirds of the molecules. The sequence of the first 200–250 amino acids appeared to be quite species specific, displaying homology of only borderline significance (Figure 2A and B). Closer examination of the N-terminal region of all three proteins revealed a hydrophilic region containing a striking cluster of SP and TP residues (Figure 1). These sites are similar to the target sequences of the $p34^{cdc2}$ protein kinase which is known to phosphorylate the B subunit of human DNA pol α in a cell cycle dependent fashion (Nigg, 1991).

The B subunit of human DNA pol α was expressed in bacteria as a glutathione S-transferase (GST) fusion protein (Smith and Johnson, 1988). The identity of the recombinant protein as the B subunit was confirmed in two ways: (i) cleavage of the fusion protein with thrombin to remove the GST domain yielded a protein product that co-migrated in SDS–PAGE with subunit B of DNA polymerase α–primase purified from HeLa cells and (ii) a monoclonal antibody raised against the fusion protein recognized subunit B of HeLa DNA polymerase α–primase (data not shown). The subunit B fusion protein, purified by adsorption to GST beads, did not manifest enzymatic activity when tested in DNA polymerase, DNA primase or protein kinase assays.

Subunit B of DNA pol α binds SV40 large T antigen

To explore the possible interactions of the B subunit with other proteins involved in initiation of DNA replication, we performed a series of co-immunoprecipitation experiments. As shown in Figure 3A, the subunit B fusion protein (lanes 1–4), but not GST alone (lanes 5–8), co-precipitated with T antigen in the presence of an anti-T antigen monoclonal antibody. Similarly, T antigen co-precipitated with the subunit B fusion protein (Figure 3B, lanes 1–4), but not GST alone (lanes 5–8), in the presence of glutathione beads. In the latter case, the failure of GST alone to precipitate T antigen was not due to poor binding of GST to the glutathione beads since ~80% of the input GST was present in the pellet (data not shown). These data indicate that the B subunit of human DNA pol α interacts specifically with the SV40 T antigen. The interaction occurs at comparable concentrations of the two proteins (see Figure 3A and B) and is relatively efficient since in both types of experiments the co-precipitated protein represented up to 20% of the input. Finally, as expected from previous studies of the interaction between T antigen and the DNA polymerase α–primase complex (Smale and Tjian, 1986; Gannon and Lane, 1987, 1990; Collins and Kelly, 1991), the observed co-precipitation was inhibited by anti-T antigen monoclonal antibody PAb205 and by murine p53 (data not shown).

In order to identify which part(s) of the B subunit is essential for T antigen binding activity, a set of deletion mutants of the fusion protein was constructed (Figure 4A). Following incubation with T antigen, each of the mutant proteins was precipitated by addition of glutathione beads, and the extent of T antigen binding was assessed (Figure 4B). Mutant proteins lacking the N-terminal 238 amino acids of

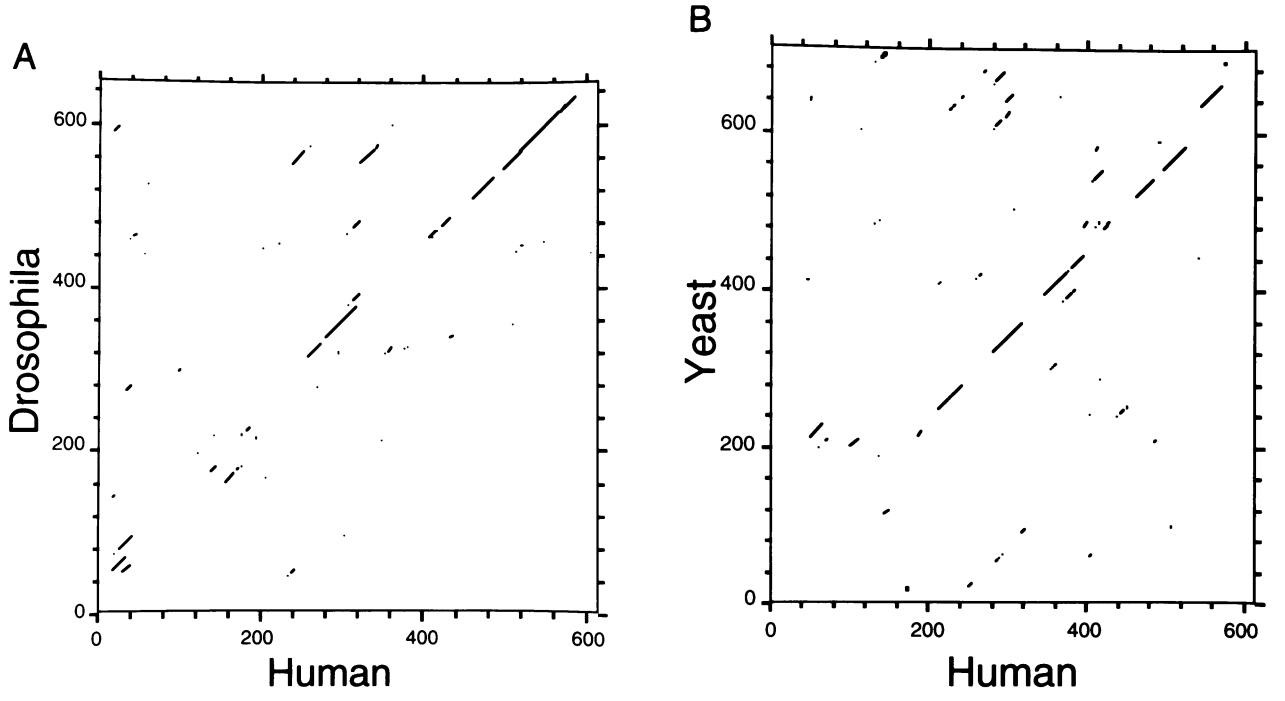

Fig. 2. Dot matrix comparisons of amino acid sequences from other species. (**A**) Comparison between human and *D.melanogaster* subunit B. (**B**) Comparison between human and *S.cerevisiae* subunit B. The parameters used for dot matrix comparison were 50% identity in a 30 amino acid window.

the B subunit were unable to bind to T antigen, while those that retained this segment of the protein bound T antigen to a similar extent as the complete fusion protein. Furthermore, this segment of subunit B was apparently sufficient for T antigen binding, since one of the active mutant proteins lacked all amino acids C-terminal to residue

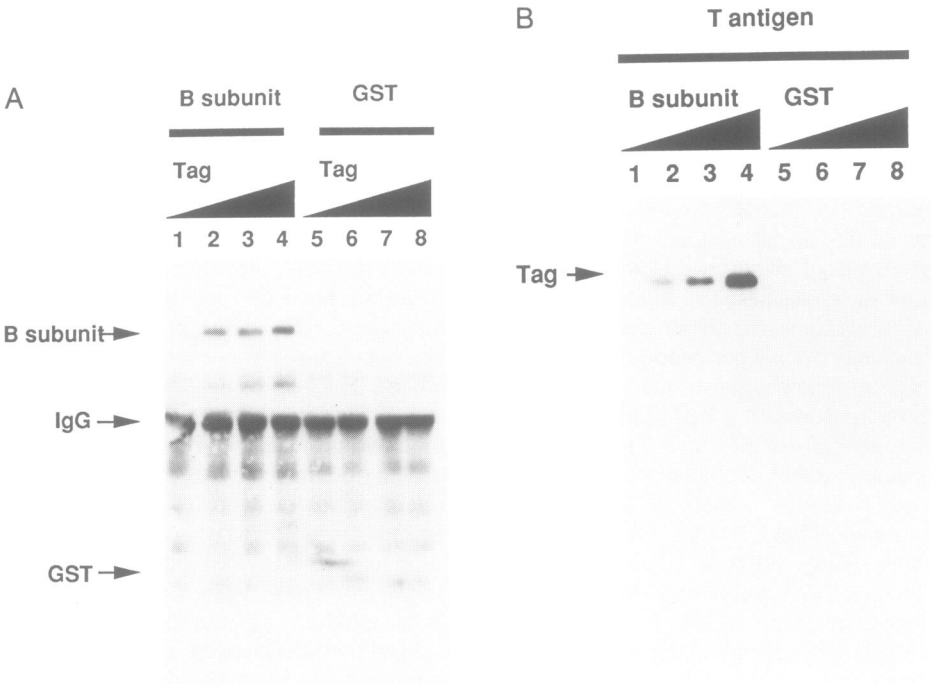

Fig. 3. Co-precipitation of subunit B fusion protein and SV40 T antigen. (**A**) 500 ng subunit B fusion protein (lanes 1–4) or 600 ng GST (lanes 5–8) was incubated with increasing amounts of T antigen (lanes 1 and 5, 0 ng; lanes 2 and 6, 200 ng; lanes 3 and 7, 400 ng; lanes 4 and 8, 800 ng). T antigen-containing complexes were precipitated by the addition of anti-T antigen monoclonal antibody PAb419 and protein A–Sepharose. The presence of co-immunoprecipitating subunit B fusion protein and GST was detected by immunoblot analysis using a polyclonal antiserum directed against the purified fusion protein. (**B**) 500 ng T antigen was incubated with increasing amounts of subunit B (lanes 1–4) or GST (lanes 5–8). Lanes 1 and 5, 0 ng; lanes 2 and 6, 250 ng; lanes 3 and 7, 500 ng; lanes 4 and 8, 1 μg. GST-containing complexes were precipitated by the addition of glutathione beads. The presence of co-precipitating T antigen was detected by immunoblot using an anti-T antigen monoclonal antibody, PAb419 (Wiekowski *et al.*, 1987).

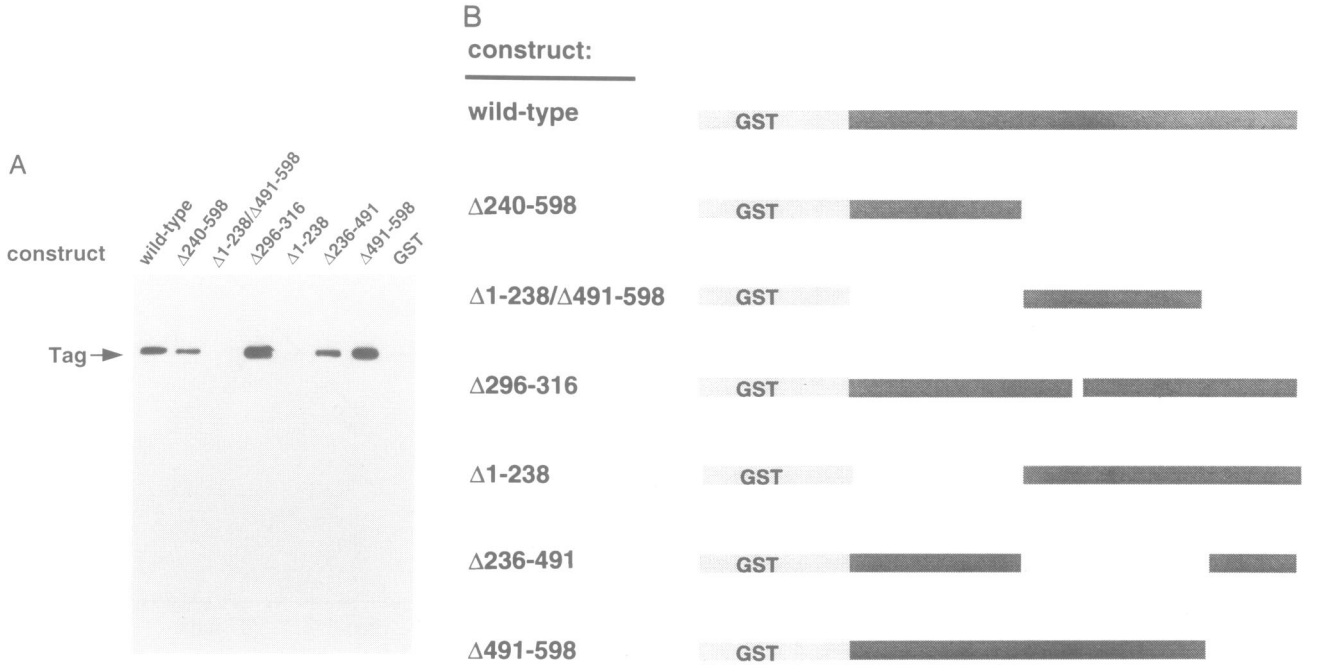

Fig. 4. Determination of the region of subunit B that binds to T antigen. (**A**) 250 ng of purified mutant or wild-type subunit B fusion protein was incubated with 400 ng SV40 T antigen. GST-containing complexes were precipitated by the addition of glutathione beads. The presence of co-precipitating T antigen was detected by immunoblot using the anti-T antigen monoclonal antibody, PAb419. (**B**) Diagrammatic representation of deletion mutants of subunit B fusion protein.

239 (Figure 4A, lane 2). The observed differences in T antigen binding activity of the various mutants were not secondary to variation in the binding to glutathione beads since all of the mutant proteins precipitated equally well (data not shown). Our conclusion that the N-terminal 239 amino acids of the B subunit are sufficient for interaction with T antigen was confirmed by reciprocal experiments in which the extent of the interaction was determined after immunoprecipitation of T antigen with a specific monoclonal antibody (data not shown).

Subunit B binds the catalytic subunit of DNA polymerase α

The 180 kDa subunit (subunit A) of DNA polymerase α–primase complex contains the DNA polymerase catalytic activity of the enzyme. Analysis of yeast DNA pol α has provided evidence for a physical interaction between the A and B subunits (Brooke *et al.*, 1991). Since the catalytic subunit of human DNA pol α has recently been cloned and expressed in active form in a baculovirus expression system (Wong *et al.*, 1988; Copeland and Wang, 1991), we were able to ask whether a similar interaction occurs in the case of human DNA pol α. We readily detected co-precipitation of subunit B with the catalytic subunit in the presence of a monoclonal antibody against the latter (data not shown). However, we were unable to perform the reciprocal experiment because monoclonal antibodies directed against the catalytic subunit of human DNA pol α were only weakly reactive in immunoblots. To overcome this problem we made use of a modified ELISA assay which has been used extensively in previous experiments to detect protein–protein interactions between replication proteins (Dornreiter *et al.*, 1990). In this assay, subunit B was immobilized in the wells of an ELISA plate. Following a blocking step to prevent subsequent non-specific protein binding, increasing quantities of the catalytic subunit were added to the wells. After washing to remove unbound protein, the presence of the catalytic subunit was detected and quantified using monoclonal antibody SJK237. As shown in Figure 5 the catalytic subunit of human DNA pol α bound the B subunit. The observed interaction between the two proteins was specific since no binding was observed when BSA was substituted for the B subunit. None of the deletion mutants of the B subunit, described above, was capable of binding to the catalytic subunit, suggesting that regions of the B subunit essential for interaction with subunit A may be rather widely distributed in the protein.

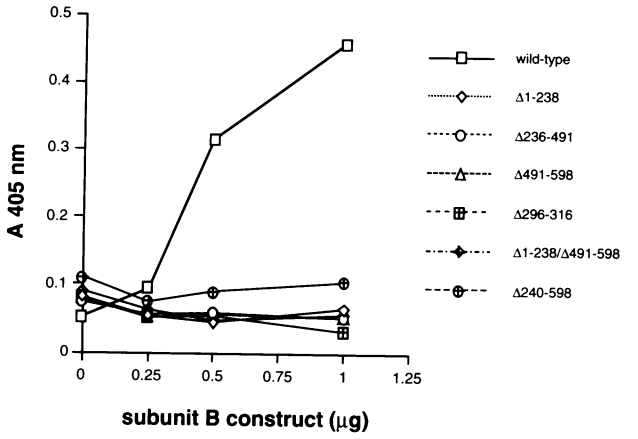

Fig. 5. Subunit B fusion protein interacts with the 180 kDa catalytic subunit of DNA polymerase α. The indicated amount of mutant or wild-type subunit B fusion protein was non-specifically bound to an ELISA plate. The wells were blocked with BSA and 0.01 units of subunit A (Copeland and Wang, 1991) was added to all the wells. After washing, retention of subunit A was detected with anti-subunit A monoclonal antibody SJK237 (Wong *et al.*, 1986).

Subunit B links T antigen and DNA polymerase α catalytic subunit

Given that subunit B of DNA pol α binds to T antigen and to the catalytic subunit individually, it was of interest to determine whether it could bind both proteins simultaneously. For this purpose T antigen was immobilized in the wells of an ELISA plate. Following a blocking step to prevent non-specific binding of the subsequently added reagents, subunit B and the catalytic subunit of DNA pol α were added sequentially. After extensive washing, the bound catalytic subunit was detected using an enzyme-linked immunoassay and specific monoclonal antibody. As shown in Figure 6A, binding of the catalytic subunit of DNA pol

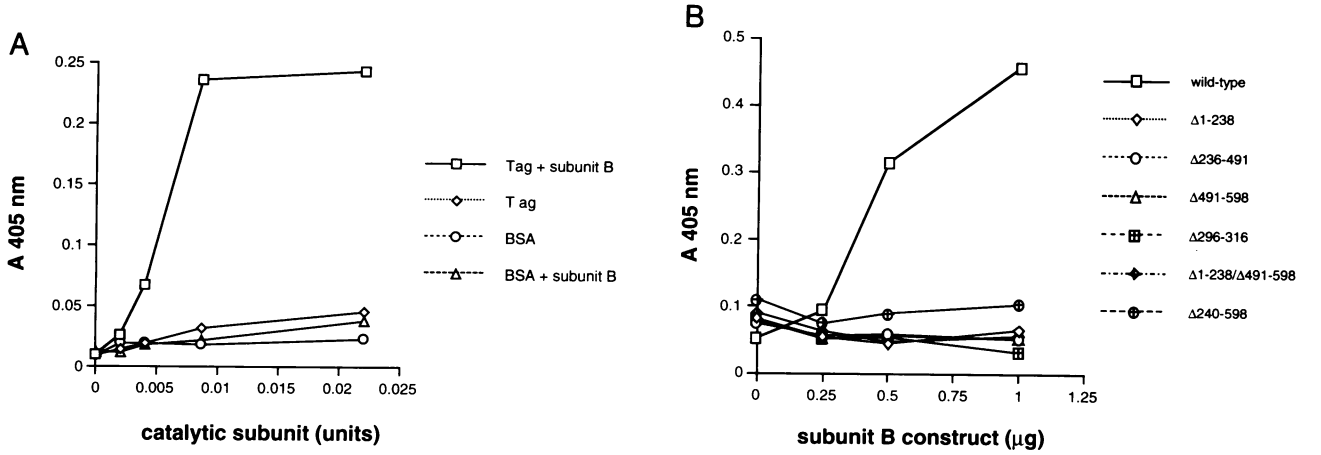

Fig. 6. Subunit B fusion protein mediates complex formation between subunit A and T antigen. (**A**) T antigen (2.0 μg) was non-specifically bound to the indicated wells of an ELISA plate. After a blocking step, 2 μg of subunit B fusion protein were added to the indicated wells. Next, subunit A was titrated into the indicated wells, and its retention after washing was detected with monoclonal antibody SJK237. (**B**) T antigen was bound to an ELISA plate as in (A). After blocking, the indicated amount of wild-type or mutant subunit B fusion protein was added to each well. Finally, 0.02 units of subunit A were added to all the wells and its retention was assayed as described in (A).

α to immobilized T antigen was readily detected, but such binding was completely dependent upon the presence of the B subunit. In control experiments binding of the catalytic subunit was not observed when addition of the B subunit was omitted or when BSA was substituted for T antigen. The simplest interpretation of these data is that the B subunit tethers the catalytic subunit to T antigen by interacting simultaneously with both proteins. We expected that, if this hypothesis were correct, none of the B subunit deletion mutants described above would be capable of serving as a tether, since none of them appears to be capable of forming stable complexes with the catalytic subunit. This expectation was confirmed (Figure 6B). The formation of the ternary complex does not appear to require ATP binding or hydrolysis as it was unaffected by the addition of ATP and ATPγS to 1 mM (data not shown). Finally, complex formation was unaffected by the presence of DNase, ruling out the possibility that contaminating DNA plays a role in the observed interactions (data not shown).

Subunit B is required for the functional interaction between SV40 T antigen and DNA polymerase α

We have previously demonstrated that the interaction between DNA polymerase α–primase and T antigen is functionally important. Specifically, the priming and synthesis of long DNA chains by the DNA polymerase α–primase holoenzyme is dramatically stimulated by the presence of T antigen (Collins and Kelly, 1991). Since the B subunit mediates a physical interaction between these two proteins, we hypothesized that it was also required for the stimulation of the activities of DNA polymerase α–primase complex by T antigen. In order to test this possibility, we examined the effect of T antigen and subunit B on the polymerase activity of the isolated catalytic subunit. The assay for polymerase activity involved extension of a short DNA primer annealed to single-stranded M13 DNA. Under the conditions of these experiments we observed that the catalytic subunit alone was capable of extending the pre-existing primer to only a limited extent, forming very small products (Figure 7A and B). The addition of subunit B alone did not detectably increase the size of DNA chains synthesized. This is in agreement with extensive biochemical analysis indicating that the isolated human catalytic subunit and the four subunit DNA pol α holoenzyme do not differ significantly in processivity, thermostability or kinetic parameters of deoxynucleoside triphosphate incorporation (Copeland and Wang, 1991). Similarly, the addition of T antigen alone had little effect on the activity of the catalytic subunit. However, we observed a dramatic stimulation in the quantity and size of DNA products when both subunit B and T antigen were added together (Figure 7A). This effect is specific since the B subunit deletion mutants which were unable to interact with the catalytic subunit were likewise unable to support T antigen stimulation. The largest stimulation was observed at relatively low concentrations of the catalytic subunit, consistent with our previous studies of the stimulation of DNA pol α holoenzyme by T antigen (data not shown) (Collins and Kelly, 1991).

In order to confirm that the stimulatory activity that we observed was due to the subunit B fusion protein and not to some contaminating bacterial protein, we further purified our preparation over a Superose 12 column. As demonstrated in Figure 8A and B, T antigen-dependent stimulation of

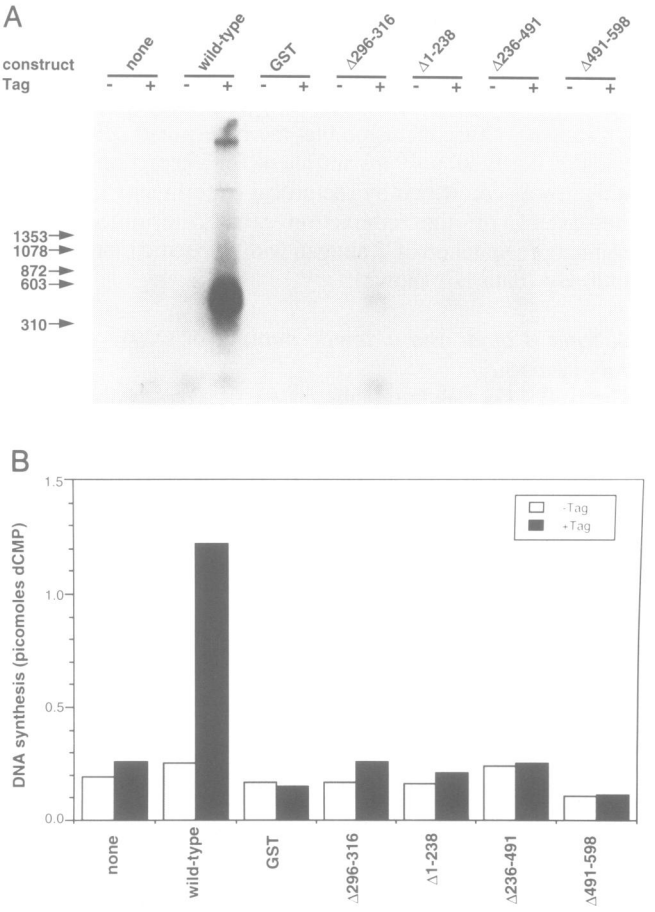

Fig. 7. Subunit B mediates T antigen stimulation of DNA polymerase α activity. (**A**) Reaction mixtures (25 μl) containing 0.01 U of DNA polymerase α activity, 250 ng of wild-type or mutant subunit B, 25 μM [α-^{32}P]dCTP, 100 μM each of dATP, dGTP and dTTP and 25 ng of single-stranded M13 DNA primed with the universal primer were assembled on ice with 400 ng of T antigen (where indicated). After incubation for 2 h at 37°C, samples were electrophoresed on a denaturing alkaline 2% agarose gel, dried and visualized by autoradiography. (**B**) The total DNA synthesized in each reaction was determined by cutting out and quantifying by scintillation counting each lane of the dried gel shown in (A).

DNA polymerase α exactly co-eluted with the fusion protein polypeptide. The stimulatory activity of the peak fraction was found to be dependent on the catalytic subunit, T antigen, and an annealed primer (Figure 9A and B). The four subunit holoenzyme containing endogenous subunit B was only slightly stimulated by additional subunit B in the presence of T antigen (Figure 9A and B). The small amount of stimulation we observed is presumably due to the presence of DNA polymerase α–primase complexes which lack active subunit B, possibly as a result of dissociation or denaturation during purification. These results lend further support to the likelihood that the physical interaction between T antigen and DNA polymerase α is functionally important for the *in vivo* function of DNA polymerase α–primase.

Inhibition of SV40 DNA replication by a mutant form of subunit B

In order to test the functional significance of T antigen–subunit B complex formation, we tested the effect of some of the subunit B deletion mutants in bona fide SV40 DNA replication. We reasoned that if formation of a DNA

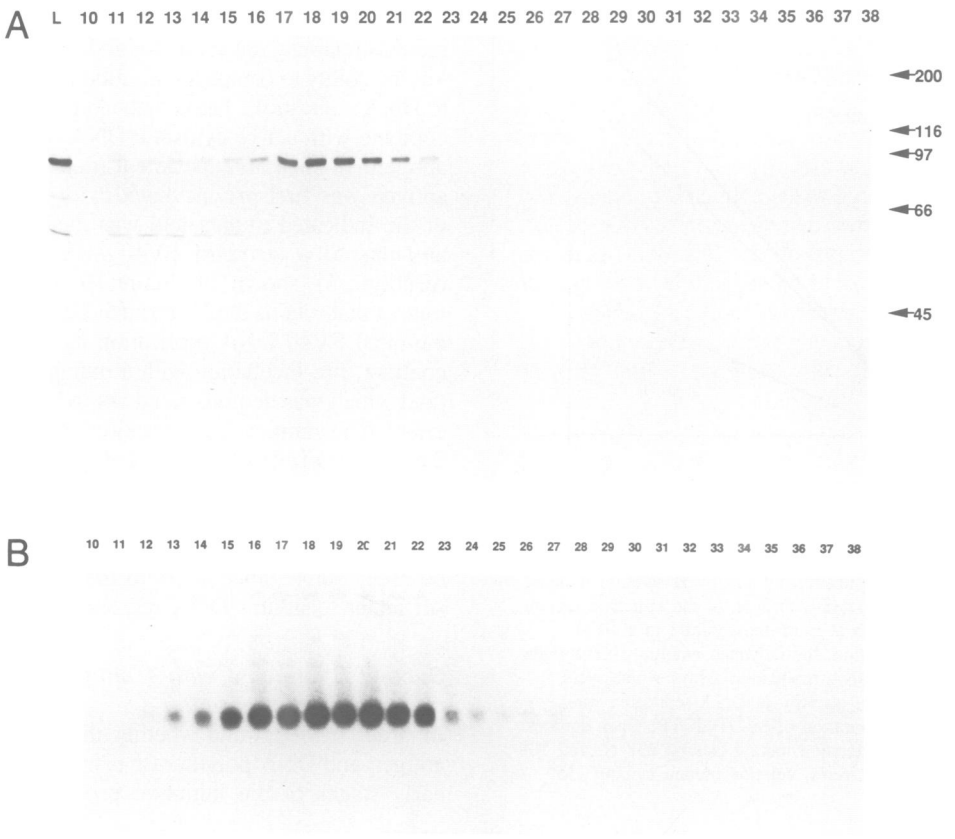

Fig. 8. Co-elution of subunit B fusion polypeptide and DNA polymerase α stimulatory activity. 140 μg of subunit B fusion protein purified by binding to glutathione−agarose were loaded on to a 25 ml Superose 12 column. Eluted samples were examined by silver stain and were assayed for their ability to activate DNA polymerase α subunit A in the presence of T antigen. (**A**) Silver stain. 10 μl of each fractions 10−33 were electrophoresed on a 10% polyacrylamide gel and silver stained. (**B**) Activity assay. Reaction mixtures (25 μl) containing 0.01 units of polymerase activity, 400 ng of T antigen, 25 ng primed single-stranded M13 DNA and 0.5 μl of the indicated column fraction were assembled on ice. Reaction products were isolated and electrophoretically separated on a denaturing agarose gel.

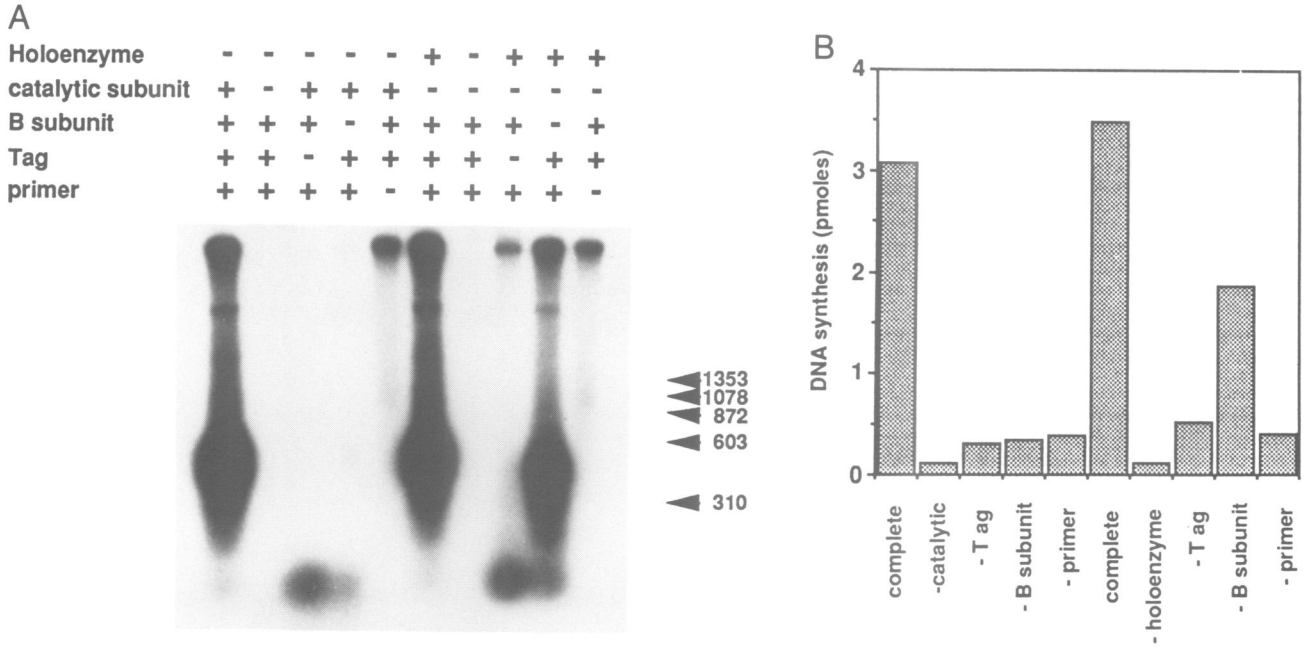

Fig. 9. Requirements for T antigen stimulation of DNA polymerase α. Reactions contained 0.01 units of either subunit A (catalytic subunit) or DNA polymerase α−primase holoenzyme (Wold *et al.*, 1989) as indicated. The complete reaction contained the components described in Figure 8, except that 0.5 μl (25 ng) of Superose fraction 19 was used. Individual components were omitted as indicated. In the reaction designated primer −, unprimed single-stranded DNA was added to the reaction. (**A**) Reaction products were visualized by alkaline agarose gel electrophoresis. (**B**) Reaction products were quantified by cutting out the lanes of the dried gel shown in (A).

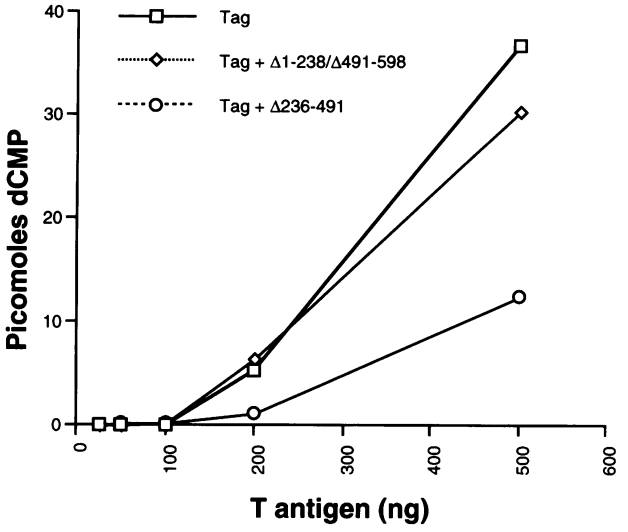

Fig. 10. Inhibition of DNA replication by subunit B deletion mutants. T antigen (1 μg) was pre-incubated with 2 μg of the indicated mutant or with buffer alone for 30 min at room temperature in a 10 μl reaction. Following pre-incubation, the indicated amount of T antigen was added to standard SV40 DNA replication assays which were incubated for 1 h at 37°C. The quantity of DNA replicated was determined by extent of incorporation of [α-^{32}P]dCTP. Open squares: buffer alone; open diamonds: deletion mutant lacking 238 N- and 108 C-terminal amino acids; open circles: deletion mutant lacking 255 internal amino acids.

pol α–T antigen complex was functionally important, mutants retaining the ability to bind T antigen but which have lost the ability to complex with subunit A should be inhibitory to DNA replication. These mutant proteins should effectively compete with active cytosolic DNA pol α for binding to T antigen. In such an experiment (depicted in Figure 10), T antigen was first pre-incubated with a 2-fold mass excess of the indicated mutant and was then added in increasing amounts to a standard SV40 *in vitro* DNA replication reaction. As shown in Figure 10, pre-incubation with a mutant that retains the N-terminal T antigen binding domain inhibited SV40 DNA replication by as much as 83%. By contrast, pre-incubation with a mutant lacking this domain (and which was demonstrated not to bind T antigen) had no effect. The simplest interpretation of this experiment is that the mutants effectively competed for T antigen binding with DNA pol α holoenzyme by forming inactive T antigen–mutant subunit B complexes, although other interpretations are possible (see Discussion). The resultant complex must be fairly stable since a 2-fold mass excess of mutant was sufficient to inhibit DNA replication dramatically.

Subunit B interacts with T antigen bound to the viral origin of DNA replication

In order to determine whether the interaction between T antigen and DNA polymerase α might be important during early stages of the initiation process, we asked whether

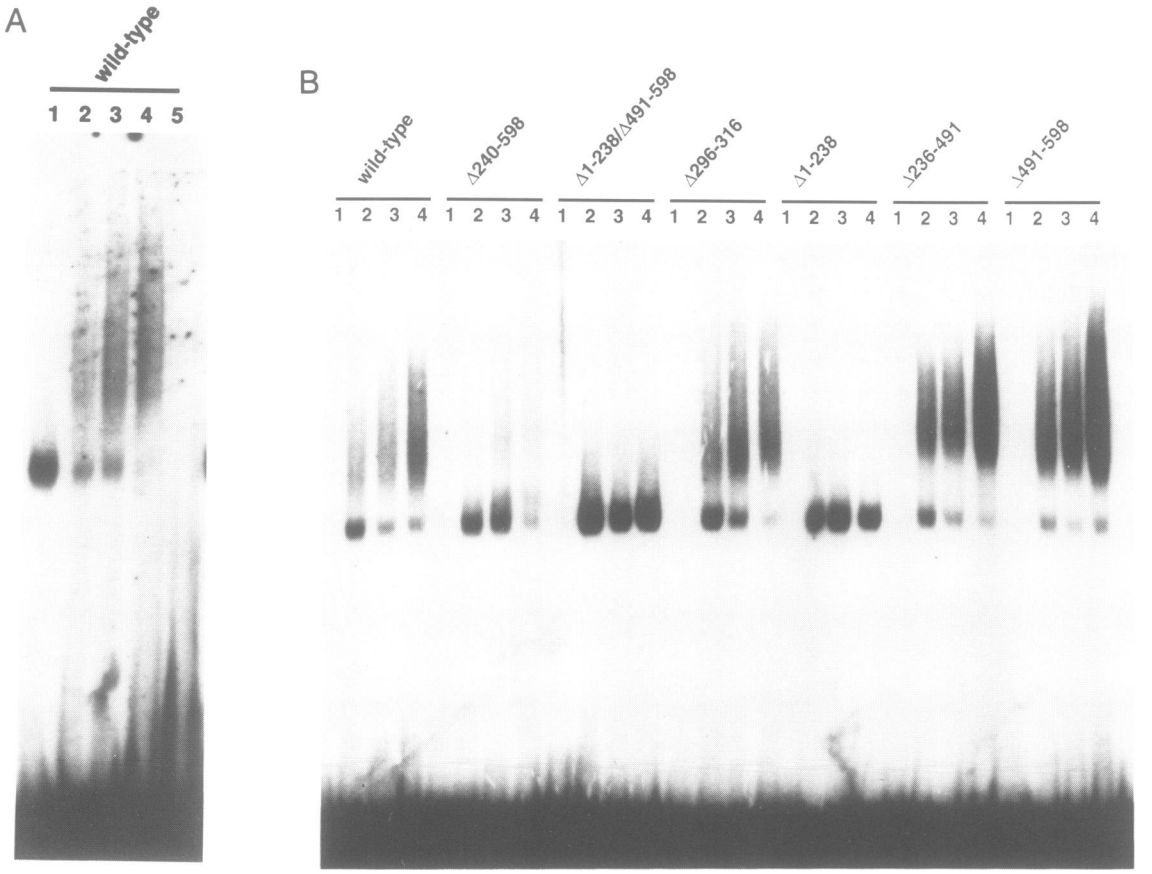

Fig. 11. Interaction of subunit B fusion protein with T antigen bound to the SV40 origin. (**A**) Gel retardation assays were performed as described in Materials and methods with 50 ng (lanes 1–4) or 0 ng T antigen (lane 5). Lanes 1–5 contain 0, 50, 100, 200 and 200 ng subunit B. (**B**) Lanes 2–4 contain 50 ng T antigen and 50, 100 and 200 ng subunit B or subunit B deletion mutant. In each case lane 1 contains 200 ng of each mutant without added T antigen.

subunit B could interact with T antigen already bound to the SV40 origin. We and others have previously demonstrated that T antigen−origin complexes can be detected in a gel retardation assay (Dean *et al.*, 1987b; Virshup *et al.*, 1992). The addition of subunit B to such an assay resulted in a supershift of these protein−DNA complexes (Figure 11A). The supershifted complexes are heterogeneous in mobility, possibly as a result of the binding of variable numbers of B subunit molecules to the double hexamer of T antigen at the origin. Additional evidence that the altered mobility of the T antigen−origin complexes is due to a direct physical interaction between T antigen and the B subunit was obtained by examining the properties of the B subunit deletion mutants. Only those deletion mutants which interact with T antigen in co-precipitation experiments are able to cause the observed supershift (Figure 11B).

Discussion

We have cloned and over-expressed the B subunit of human DNA polymerase α as a fusion protein in bacteria. The fusion protein was shown to form a stable complex with T antigen in solution and with T antigen bound to the SV40 origin of replication. We also demonstrated that subunit B promoted formation of a complex containing both T antigen and the catalytic subunit (subunit A) of DNA pol α. These findings are consistent with the idea that subunit B acts as a molecular tether linking the two proteins. It seems likely that this complex is important for efficient SV40 DNA replication, since the ability of T antigen to enhance the synthesis of long DNA chains by the catalytic subunit was highly dependent upon the presence of the B subunit.

The region of the B subunit which interacts with T antigen has been localized to the N-terminal 239 amino acids. Analysis of the amino acid sequence in this domain reveals a segment of ~50 amino acids which is rich in proline, serine and threonine (seven, 10 and six residues respectively; Figure 1, residues 115−157). The hydrophilicity of the segment suggests that it resides on the surface of the protein and the frequency of α- and β-breaking residues suggests that it may be devoid of secondary structure. Within the sequence is a cluster of possible phosphorylation sites for the cell cycle dependent $p34^{cdc2}$ protein kinase. The location of this segment in the region required for binding to T antigen suggests that phosphorylation may regulate the ability of the DNA polymerase α−primase complex to bind to the cellular replication machinery. For example, this unstructured portion of the binding region may form a kind of hinge which could be regulated to expose the interaction domain depending on its phosphorylation state. It has previously been reported that the B subunit is a phosphoprotein and that its state of phosphorylation varies during the cell cycle (Nasheuer *et al.*, 1991). The protein appears to be largely unphosphorylated in G_1 and S phases, but becomes phosphorylated following completion of DNA replication. *In vitro* the B subunit is a substrate for the $p34^{cdc2}$ protein kinase, and the residues that are phosphorylated by the enzyme are the same as those phosphorylated *in vivo* (Nasheuer *et al.*, 1991). Experiments aimed at determining whether phosphorylation by cdc2 kinase affects the ability of the B subunit to mediate complex formation between T antigen and the DNA polymerase α catalytic subunit are in progress.

Under the conditions of our experiments we did not detect a direct physical interaction between T antigen and the catalytic subunit of DNA polymerase α in the absence of the B subunit. In previous work Dornreiter *et al.* (1990) reported detection of such an interaction using a modified immunoblotting technique. In these experiments the four subunits of the DNA polymerase α−primase complex were separated by SDS−PAGE and then renatured *in situ* prior to incubation with T antigen. More recent studies have provided evidence that the domain of the catalytic polypeptide capable of interacting with T antigen resides in the N-terminal region of the protein (Dornreiter *et al.*, 1993). Such a direct interaction between the catalytic subunit of DNA pol α and T antigen presumably contributes to complex formation, but our data indicate that the formation of a highly stable and functionally significant complex is greatly enhanced by the presence of the B subunit. Under some conditions, particularly at relatively high T antigen concentrations, we observed a weak stimulation of the DNA polymerase activity of the catalytic subunit by T antigen in the absence of subunit B. However, under all conditions that we tested, maximal polymerase activity on a primed single-stranded DNA template required both T antigen and subunit B. It is unclear why binding of T antigen to the B subunit was not detected in the immunoblotting assay (Dornreiter *et al.*, 1990). However, one reasonable possibility is that renaturation of the B subunit after SDS−PAGE and electroblotting is inefficient.

Our experiments indicate that the binding of subunit B to the catalytic subunit of DNA pol α does not of itself increase the ability of the enzyme to synthesize long DNA chains. This result is consistent with results from studies of *S.cerevisiae* DNA polymerase I. In those studies isolated subunit B was shown to form a stable complex with the isolated catalytic subunit *in vitro* (Brooke *et al.*, 1991). When compared with the catalytic subunit alone, the binary complex exhibited a greater thermal stability and an increased rate of association with primase subunits. However, binding of subunit B to the catalytic subunit did not affect any of the parameters of catalysis such as the processivity, the K_m for dATP or the K_m for the primer terminus (Brooke and Dumas, 1991).

In this and previous studies, we have demonstrated that the ability of DNA pol α to prime the synthesis of new DNA chains and to extend pre-existing DNA chains is dramatically enhanced by the presence of SV40 T antigen (Collins and Kelly, 1991; Erdile *et al.*, 1991). The finding that this stimulatory effect requires subunit B is consistent with a model in which subunit B promotes synthesis by directly coupling DNA pol α to T antigen (Collins and Kelly, 1991). In this model, the linkage of DNA pol α to T antigen tethers the polymerase to the DNA template, thus increasing the efficiency of priming and elongation. DNA polymerase α is normally not a very processive enzyme, synthesizing about 7−13 nucleotides per binding event and then dissociating from the DNA terminus (Copeland and Wang, 1991). On the other hand, T antigen binds stably to single-stranded DNA under physiological conditions and, in the presence of ATP, is able to translocate in the 3′ to 5′ direction. Thus, the tethering of DNA pol α to T antigen would be expected to hold the polymerase within the domain of the DNA template after the polymerase dissociates from the terminus of a newly synthesized DNA strand. The rate of reassociation

of the tethered polymerase with the template would be greatly enhanced over that of a polymerase molecule which had left the domain of the DNA and diffused into the bulk solution. Such a tethering model would explain our observation that both the total amount of DNA synthesized and the average chain length increase when T antigen and subunit B are present. The increase in the total amount of DNA synthesis is due to a more rapid association with the template as a result of the close proximity of the DNA pol α to the DNA template. The increase in chain length is a result of the fact that the tethered polymerase preferentially extends the same local DNA strand. This general model is also in agreement with the observation that the stimulatory effect of T antigen is greater at limiting DNA and polymerase concentrations (Collins and Kelly, 1991). An alternative model to explain our functional data is that the interaction of subunit B with the isolated subunit A serves to stabilize the latter. Stabilization of the A subunit could directly increase apparent polymerase activity or could enhance the ability of the A subunit to interact productively with T antigen. Although we cannot rule out this stabilization model, it is somewhat less appealing since the B subunit has relatively little effect on the activity of the isolated subunit A in the absence of T antigen.

The finding that a mutant form of subunit B inhibits SV40 DNA replication *in vitro* is consistent with the hypothesis that the interaction between subunit B of DNA pol α and T antigen is important in DNA replication. Our data do not indicate which step in DNA replication is inhibited by the mutant subunit B. However, one reasonable interpretation is that the mutant protein interferes with the normal interaction between T antigen and DNA pol α. The tethering of DNA pol α to T antigen could play a critical role during both the initiation and elongation phases of the reaction. We have shown that the B subunit can interact with T antigen that is bound to the viral replication origin. Thus, the formation of a complex between T antigen and DNA pol α may be one of the earliest steps in the initiation reaction. The recruitment of DNA pol α to the proximity of the origin may greatly facilitate priming and synthesis of the first DNA chains following the local unwinding of the duplex (see Figure 12). During the subsequent elongation phase of the replication reaction, the T antigen–DNA pol α complex constitute a helicase–primase machine that is in position to synthesize primers efficiently on newly exposed DNA strands as the fork progresses. The model shown in Figure 12 provides a mechanism for how the primase-helicase complex promotes efficient repetitive rounds of Okazaki fragment synthesis. This is accomplished by continued association of DNA pol α with T antigen throughout Okazaki fragment synthesis, thus preventing it from diffusing into the bulk solution after dissociation from the terminus of the nascent DNA strand.

To date much of our understanding of eukaryotic DNA replication has been obtained indirectly through the study of viruses such as SV40. Given that the mechanism of SV40 DNA replication appears to be similar to that of cellular DNA replication, we can speculate that cells contain factors that act in a similar manner to SV40 T antigen. In *S.cerevisiae* a factor has been identified that specifically recognizes *S.cerevisiae* origins of replication (Bell and Stillman, 1992). It has yet to be determined whether this

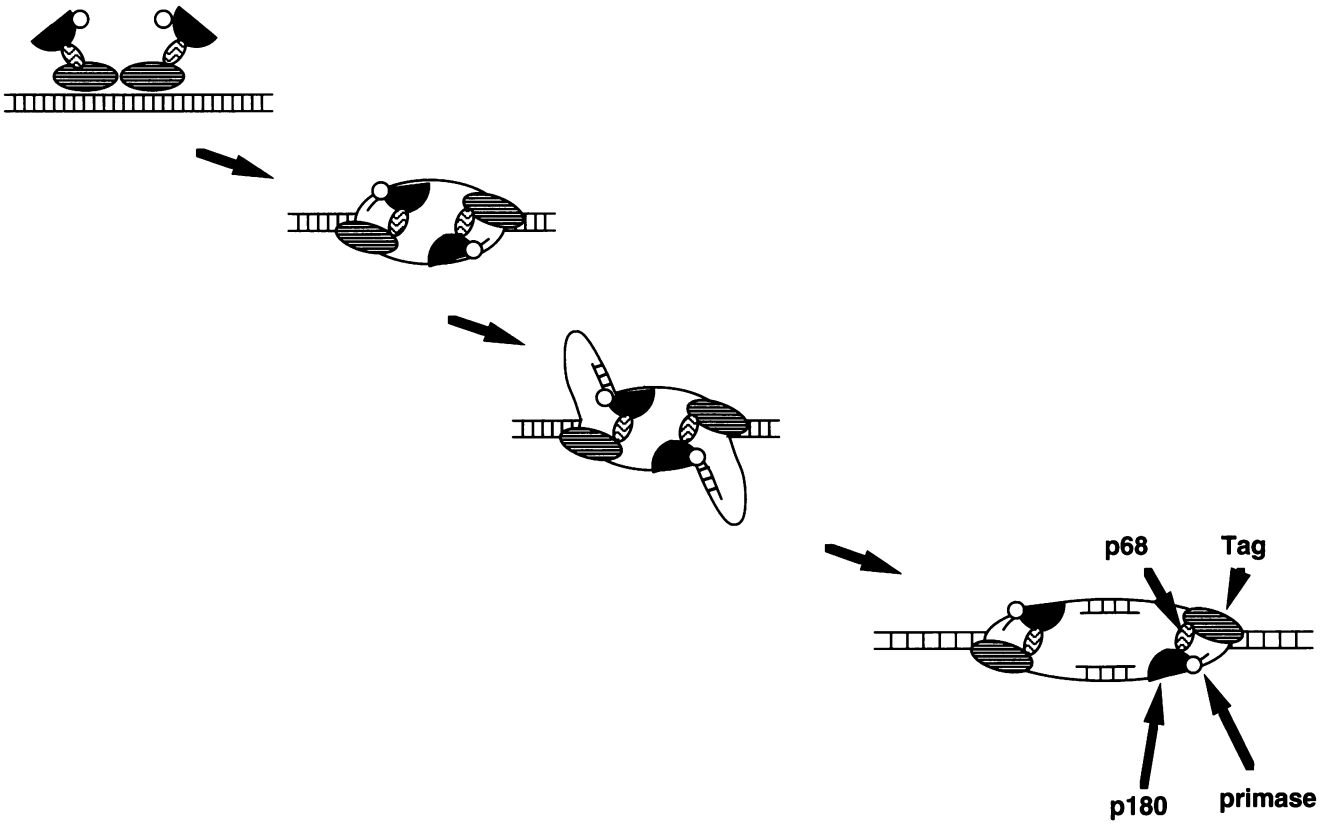

Fig. 12. Model for the role of subunit B in DNA synthesis. T antigen unwinds the DNA at the origin and then functions as a helicase, moving 3′–5′ on the template strand. DNA polymerase α–primase complex synthesizes a primer and extends it 5′–3′. The B subunit tethers these activities and promotes multiple rounds of Okazaki fragment synthesis (see text for further details).

multi-enzyme complex shares other activities with T antigen such as recruiting and activating DNA pol α. Unfortunately in higher eukaryotes there are no clearly elucidated origin sequences that can be used to identify similar origin binding factors. However, since human subunit B tightly associates with the SV40 origin binding protein, it may represent a powerful affinity reagent to identify human proteins that act analogously to T antigen in the initiation and/or elongation of DNA chains.

Materials and methods

Cloning and expression of subunit B in bacteria

Murine DNA polymerase α was purified (Prussak and Tseng, 1987) and the amino acid sequence of three subunit B peptides was obtained (Prussak *et al.*, 1989): p68.7, VVGDPEPLTGSYK; p68.18, EYSLFPGQVVIMEGFNTTGR; and p68.20, DIVSIQELIEAEEEEETLLSSYTXPSK. Degenerate oligonucleotide primers were made to the six N- and C-terminal amino acids of p68.18 and p68.20 and were used in PCRs to generate DNA fragments from murine cDNA as described previously (Bredt *et al.*, 1991). Briefly, these fragments were cloned and the exact nucleotide sequence of the non-degenerate portion of the fragment was determined. This sequence was used to generate exact sequence oligonucleotide primers which were used in a PCR to amplify a 629 nucleotide fragment from murine cDNA. This cDNA was sequenced and used to probe a HeLa λgt 11 cDNA library. Two human cDNA clones were isolated from 400 000 plaques screened. The cDNA was sequenced on both strands using the Sanger dideoxy chain termination method.

The cDNA encoding the 66 kDa subunit of human DNA polymerase α was cloned into pGEX 2T (Promega) and transformed into *Escherichia coli* CAG cells. The fusion protein was purified as described by Smith and Johnson (1988), with some modifications. The expressed protein was mostly insoluble and the soluble material was highly unstable. Recovery of full-length protein was increased by omitting IPTG induction and recovering the protein from an overnight culture. Bacterial cell pellets were frozen in liquid nitrogen, thawed and resuspended in 1/20 volume of buffer A [1 × PBS, 1 mM dithiothreitol (DTT), 0.25 mM EDTA and 0.2 mM PMSF]. The resuspended material was sonicated twice for 30 s at a setting of 4 on a Branson Sonifier 250. Following sonication, Triton X-100 was added to a concentration of 1%. Insoluble material was removed by centrifugation at 17 000 *g* for 5 min. The fusion protein was bound to glutathione beads in batch at 4°C for 30 min. The beads were washed three times in buffer A and twice in buffer B (100 mM Tris, 50 mM NaCl, 1 mM DTT, 0.2 mM PMSF, pH 8). Fusion protein was eluted by incubation with buffer B containing 10 mM glutathione and was dialyzed into buffer C (30 mM HEPES, pH 7.8, 25 mM KCl, 0.25 mM EDTA, 0.01% NP40, 1 mM DTT, 0.25% inositol). Approximately 0.3 mg was recovered from a 500 ml cell culture. For activity assays, the fusion protein was taken through a second round of binding to the beads. Routinely only ~10–30% of the fusion protein bound glutathione beads a second time.

Construction of subunit B deletion mutants

Large deletion mutants were constructed by standard cloning techniques, taking advantage of naturally occurring restriction enzyme sites. The small deletion of amino acids 296–316 was made using PCR as described by Higuchi (1990). All mutants were purified with a wild-type control exactly as described above.

Purification of DNA polymerase α

DNA polymerase α holoenzyme was purified as described by Wold *et al.* (1989). The catalytic subunit of DNA polymerase α was purified essentially as described by Copeland and Wang (1991) from recombinant baculovirus AcHDPα (generously provided by T.S.-F.Wang). Modifications in the published purification were as follows. The SJK237-71 monoclonal antibody column was prepared by covalent crosslinking to Affi-gel 10 matrix (Bio-Rad). The catalytic subunit was eluted from this column with 50% (v/v) ethylene glycol, 0.5 M KCl, 20 mM Tris–HCl (pH 8.5), 1 mM EDTA, 10% (v/v) glycerol, 1 mM 2-mercaptoethanol and 0.2 mM PMSF. The immuno-affinity eluate, which contained trace amounts of antibody light chain, was dialyzed into 50% (v/v) glycerol, 20 mM Tris–HCl (pH 8.5), 1 mM EDTA, 50 mM KCl, 1 mM DTT and 0.2 mM PMSF, and stored at −20°C. Enzyme activity was determined as described by Wold *et al.* (1989). Under our standard assay conditions the specific activity of the purified A subunit was ~7500 units per mg and was comparable to that of highly purified HeLa DNA polymerase α holoenzyme.

Co-precipitation assays

The indicated amounts of T antigen and subunit B were incubated in a 25 μl reaction containing IP reaction buffer (30 mM HEPES, pH 7.8, 7 mM $MgCl_2$, 1 mM DTT, 40 mM creatine phosphate and 4 μg BSA) for 30 min at room temperature. For precipitation of T antigen with subunit B, 50 μl of a 50% solution of glutathione beads in wash buffer (30 mM HEPES, pH 7.8, 0.25% inositol, 0.25 mM EDTA, 0.01% NP40, 1 mM DTT, 15 mM KCl, 7 mM $MgCl_2$, 40 mM creatine phosphate) was added and rotated for 1.5 h at 4°C. For precipitation of subunit B with T antigen, 13 μg of anti-T antigen monoclonal antibody was added and incubated for 30 min on ice. Next 50 μl of a 50% solution containing protein A (Pharmacia) in wash buffer was added and samples were rotated for 1 h at 4°C. The supernatants were recovered and the pellets were washed three times with IP wash buffer. Samples were boiled in Laemmli buffer, electrophoresed on 10% SDS–PAGE and transferred to nitrocellulose. Immunoblots were probed using ECL (Amersham) as described by the manufacturer.

ELISA assays for detection of protein–protein interactions

ELISAs were performed essentially as described (Dornreiter *et al.*, 1990) with some modifications. The indicated amount of first antigen was bound to the ELISA plate (NUNC, Maxisorp) in a total volume of 25 μl buffer C at room temperature for 1 h. Wells were then blocked for 2 h at room temperature with a solution containing 5% BSA in IP wash buffer. Next the second antigen was added in a 25–30 μl reaction volume containg IP reaction buffer. (If a third antigen was to be added a second blocking step was included for 30 min at room temperature and the third antigen was added in a 25 μl reaction volume containing IP reaction buffer.) Primary antibody (SJK237) was added next (1 μg in 25 μl IP wash buffer with 5 μg BSA) and incubated either overnight at 4°C or for 1 h at room temperature. Secondary antibody (sheep anti-mouse, Amersham) diluted 1:250 in a solution containing IP wash buffer with 3% BSA was next added in a 25 ml volume. Reactions were developed exactly as described (Dornreiter *et al.*, 1990). In between each step wells were washed three times with IP wash buffer. Unless otherwise indicated reactions were incubated for 1 h at room temperature.

T antigen stimulation of DNA polymerase α

Reactions were carried out as described by Collins and Kelly (1991). Briefly, 25 μl reactions were assembled on ice and contained 25 ng M13 mp18 single-stranded DNA annealed to the universal sequencing primer, 2.5 μg BSA, 0.01 units DNA polymerase activity, 400 ng SV40 T antigen prepared from HeLa cells expressing T antigen in an adenovirus vector (Wold *et al.*, 1989) [some preparations of T antigen expressed in baculovirus contained contaminants (probably nuclease) that caused primer-independent DNA synthesis], the indicated amount of subunit B fusion protein, 100 μM each dATP, dTTP and dGTP, 25 μM dCTP, [α-^{32}P]dCTP, 30 mM HEPES pH 7.8, 7 mM $MgCl_2$, 40 mM creatine phosphate and 100 μg/ml creatine kinase. Reactions were carried out for 2 h at 37°C and samples were precipitated to remove unincorporated radioactivity. Reaction products were analyzed by alkaline agarose gel electrophoresis and autoradiography.

Superose column purification

140 μg of purified subunit B fusion protein was loaded on to a 25 ml Superose 12 column (Pharmacia) which had been pre-equilibrated in buffer C containing 50 mM KCl. Fractions (600 μl for the first 12 fractions, 300 μl thereafter) were collected for about 3 column volumes. Samples were assayed for protein content (Bio-Rad protein assay kit) using BSA as a standard. Essentially all of the protein was eluted in the first 33 fractions. These fractions were analyzed by silver stain and were assayed for their ability to support T antigen stimulation.

Inhibition of SV40 DNA replication

Pre-incubation reactions contained 2 μg of the indicated mutant, 1 μg T antigen, 30 mM HEPES, pH 7.8, 7 mM $MgCl_2$ and 40 mM creatine phosphate. Reactions were incubated at room temperature for 30 min and titrated into standard SV40 replication reactions (Wold *et al.*, 1989). DNA synthesis was quantified by incorporation of [^{32}P]dCTP into DNA as measured by TCA precipitation.

Gel mobility shift assays

Reactions were performed essentially as described (Virshup *et al.*, 1992) with the following modifications: DNA retardation mixes contained 3 fmol ^{32}P-labelled 67 bp SV40 minimal origin DNA, 50 ng SV40 large T antigen purified from an adenovirus expression system (Wold *et al.*, 1989), 0–200 ng subunit B protein in 30 mM HEPES (pH 7.8), 7 mM $MgCl_2$, 40 mM creatine phosphate, 1 mM dithiothreitol (DTT), 2.5 μg acetylated bovine serum albumin and 1 mM adenyl-imidophosphate (AMP-PNP). Binding

mixes were assembled on ice with all the components added except the T antigen. Reactions were initiated by shifting the tubes to 37°C and adding T antigen to a final volume of 10 μl. After 30 min, the reactions were crosslinked by the addition of glutaraldehyde to 0.5% final concentration at 37°C for 5 min. The complexes were then electrophoresed and analyzed as described by Virshup *et al.* (1992).

Acknowledgements

We thank Dennis Wilson for production of monoclonal antibodies directed against the subunit B fusion proteins, Drs Jef Boeke, Dan Herendeen and Ellen Fanning for critical reading of the manuscript, and Dr Randy Reed and Dr Lonny Levin for useful scientific advice. We thank Dr Daniel Herendeen for providing purified DNA polymerase α catalytic subunit. We are grateful to David Hinkle for providing the unpublished sequence of *S.cerevisiae* subunit B. K.L.C. is the recipient of a Medical Scientist Training Program award and research was supported by grants PO1 CA16519 and RO1 CA40414.

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Received on May 18, 1993; revised on August 26, 1993

Note added in proof

The nucleotide sequence data reported here have been deposited in the GenBank/EMBL data library under the accession number L24559.