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Helix-Destabilizing Properties of the Human Single-Stranded DNA- and RNA-Binding Protein Purα

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Abstract Purα is a ubiquitous nucleic acid-binding protein which has been implicated in the control of eukaryotic gene transcription. Further, Purα associates with DNA sequences positioned in close proximity to viral and cellular origins of replication suggesting a role for this protein in DNA replication. As initiation of transcription and replication require alteration in the structure of duplex DNA, we investigated the DNA unwinding activity of this single-stranded nucleic acid-binding protein. Here we demonstrate that Purα has the ability to displace an oligonucleotide annealed to single-stranded M13 DNA. The helix unwinding activity of Purα was dose-, time- and temperature-dependent and ATP-independent. Results from mapping studies revealed that the central region of Purα, spanning amino acids 72–274, was important for the helix-destabilizing activity of this protein. The region of Purα which was involved in the helix-destabilizing activity mapped to the DNA-binding domain of this protein. Results from heat inactivation experiments demonstrated that the helix-destabilizing activity of Purα correlates with its capacity to interact with DNA containing the PUR element. Taken together, these studies demonstrate that Purα possesses helix-destabilizing activity and that this activity maps to and correlates with its ability to interact with DNA. J. Cell. Biochem. 80:589–595, 2001.

Key words: Purα; single-stranded; helix-destabilization; unwinding; transcription; replication; nucleic acid

Human Purα is a 322 amino acid sequence-specific, single-stranded DNA-binding protein which is implicated in the control of both DNA replication and gene transcription [for review see Gallia et al., 2000]. Purα was initially characterized as a Hela cell nuclear protein which bound to a sequence element adjacent to a region of stably-bent DNA positioned upstream of the human c-myc gene [Bergemann and Johnson, 1992]. This element, called PUR, is near the center of a region implicated as an initiation zone for chromosomal DNA replication and is found at several eukaryotic origins of DNA replication. Further, Purα has been shown to interact with the human neurotropic JC virus (JCV) and Bovine Papillomavirus (BPV) origins of replication [Chang et al., 1996; Jurk et al., 1996]. Although the biological activity of Purα on eukaryotic or BPV replication is yet undescribed, Purα has been shown to inhibit JCV DNA replication in glial cells [Chang et al., 1996].

Purα has also been implicated in gene transcription involving several promoters including JCV early gene promoter [Chen and Khalili, 1995], myelin basic protein promoter [Haas et al., 1995], FE65 gene promoter [Zambrano et al., 1997], neuronal nicotinic acetylcholine receptor gene promoter [Du et al., 1997], vascular smooth muscle α-actin gene promoter [Kelm et al., 1997], and several other eukaryotic promoters [Hereault et al., 1993; Osugi et al., 1996; Thatikunta et al., 1997].

Purα has a modular structure which is composed of three 23 amino acid Class I repeats interspersed with two 26 amino acid Class II repeats. Each repeat, although not completely identical, possesses several identical and conservatively substituted amino acid residues. Class I repeats are composed of three conserved phenylalanine or tyrosine residues as well as

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several basic amino acids. The Class II repeats, characterized by acidic and leucine amino acid residues, are involved in the interaction between Purα and the transcriptional transactivator protein Tat, from the human immunodeficiency virus 1 (HIV-1) [Krachmarov et al., 1996]. This central repeat region of Purα is also important for its binding to single-stranded DNA target sequences [Chen et al., 1995; Johnson et al., 1995].

Purα is a member of the Pur protein family. Alignment of the predicted amino acid sequence of murine Purα and the other family members, including Purβ, showed a 71% identity [Kelm et al., 1997]. The basic 23 amino acid Class I repeats and acidic 26 amino acid Class II repeats are highly conserved between Purα and Purβ. In addition to its ability to interact with nucleic acids, Purα also associates with several transcription factors [for review see Gallia et al., 2000]. For example, earlier studies from our laboratory and others showed the interaction of Purα with cellular proteins including Sp1, E2F-1, pRb, and YB-1 [Darbinian et al., 1999; Johnson et al., 1995; Safak et al., 1999; Tretiakova et al., 1999], and several viral proteins including JCV T-antigen [Gallia et al., 1998] and HIV-1 Tat [Gallia et al., 1999; Krachmarov et al., 1996].

The ability of Purα to control transcription and replication of eukaryotic genes prompted us to investigate several activities which are involved in these processes. Here, we demonstrate the ability of Purα to unwind the DNA molecule and that the region which is important for this activity overlaps with the region which is required for its interaction with DNA.

MATERIALS AND METHODS

Plasmid Constructs and Oligonucleotides

GST-Purα, and its mutant variants which are used in this study, have been previously described [Gallia et al., 1998]. Oligonucleotides were synthesized by Express Genetics (Princeton, NJ). The nucleotide composition of the oligonucleotides used in this study is as follows:

Universal 15-mer: 5'-AGTCACGTTGACGTA-3' (for unwinding assay)
VG-1: 5'- AGCTTGGAGGCGGAGGCGGCCTCGGCG-3' (for band-shift-assay)

Unwinding Assay

The substrate utilized in unwinding assays was made by annealing the 15-mer universal sequencing primer to M13mp18 single-stranded DNA (U.S. Biochemical, Cleveland, OH). The primer was labeled with [α^{32}P] dATP using DNA polymerase (Boehringer-Mannheim, Indianapolis, IN). This resulted in a single-stranded circular DNA with an 18-bp double-stranded region. Unwinding reaction was carried out by the method described previously [Stahl et al., 1986]. Reactions were stopped by the addition of SDS to a final concentration of 0.3% and EDTA to a final concentration of 0.05 M. Samples were analyzed on 9% native polyacrylamide gels in 0.5X TBE buffer for 3 h at 160 V at room temperature.

Protein Purification

GST fusion proteins were expressed in *E. coli* and purified according to the method described previously [Gallia et al., 1999]. Briefly, bacteria were grown overnight at 37°C in a Luria Bertani medium supplemented with 100 mg/L ampicillin. After 16 h, bacteria were diluted 1:10 in fresh LB medium, grown to an OD at 595 nm of 0.6, and induced for 2 h at 37°C with 0.5 mM isopropyl-β-D thiogalactopyranoside (IPTG). Bacteria were collected by centrifugation at 7,000 × *g* at 4°C, resuspended in NETN buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40) containing 2 mg/ml lysozyme, 1 μg/ml leupeptin, 1 μg aprotinin, and 1 mM phenylmethylsulfonyl fluoride, and sonicated on ice. The bacterial lysate was centrifuged at 40,000 × *g* at 4°C. Glutathione-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ) were added to the supernatant and incubated at 4°C for 2 h. Beads were pelleted and washed three times with 25–50 volumes of NETN buffer each time. The integrity and purity of the GST fusion proteins were verified by SDS-PAGE followed by Coomassie Blue staining.

Electrophoretic Mobility Shift Assays

Oligonucleotide VG-1 was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Boehriger Mannheim, Indianapolis, IN). Bacterially produced GST-Purα or GST was preincubated for 30 min at various temperatures as indicated, and subsequently incubated with 54,000 cpm of labeled probe in a final volume of

20 μl for 1 h at 4°C. Complexes were resolved by electrophoresis in 9% native polyacrylamide gels in 0.5X TBE. Electrophoresis was carried out at 180 V for 3–4 h at 4°C. After electrophoresis, the gel was dried and exposed to x-ray film.

RESULTS AND DISCUSSION

We examined the helix-destabilizing properties of Purα on a partial duplex DNA template consisting of a single-stranded M13mp18 molecule annealed to a radiolabelled 18 base oligonucleotide. In the first series of experiments, unwinding assays were performed with increasing amounts of GST and GST-Purα. As shown in Figure 1A, incubation of GST-Purα with the partially duplex DNA substrate resulted in dose-dependent displacement of the radiolabelled single-stranded oligonucleotide (compare Lane 2 to Lanes 5–7). This activity was specific for Purα as incubation of equal molar concentrations of GST with the DNA substrate had no effect on the stability of the duplex DNA (Fig. 1A, compare Lane 2 to Lanes 3 and 4). In the next series of experiments, we investigated the requirement of ATP for Purα to induce duplex destabilization. As shown in Figure 1B, Purα was able to dissociate the 18-mer DNA probe from the single-stranded M13mp18 in the presence and absence of ATP (compare Lanes 5 and 6 to 7 and 8). These observations suggest that Purα possesses ATP-independent DNA unwinding/helix-destabilizing activity.

To further examine the helix-destabilizing property of Purα, we performed time-course and temperature-dependent experiments. In the first series of studies, an equal amount of GST-Purα was incubated at 37°C with the partially double-stranded DNA substrate for various times. As shown in Figure 2A, incubation of GST-Purα with the DNA substrate resulted in time-dependent displacement of the radiolabelled single-stranded oligonucleotide (compare Lane 2 to Lanes 3–7).

Next, we investigated the influence of temperature on the ability of Purα to separate the partial duplex DNA. Increasing amounts of Purα were incubated with the partially double-

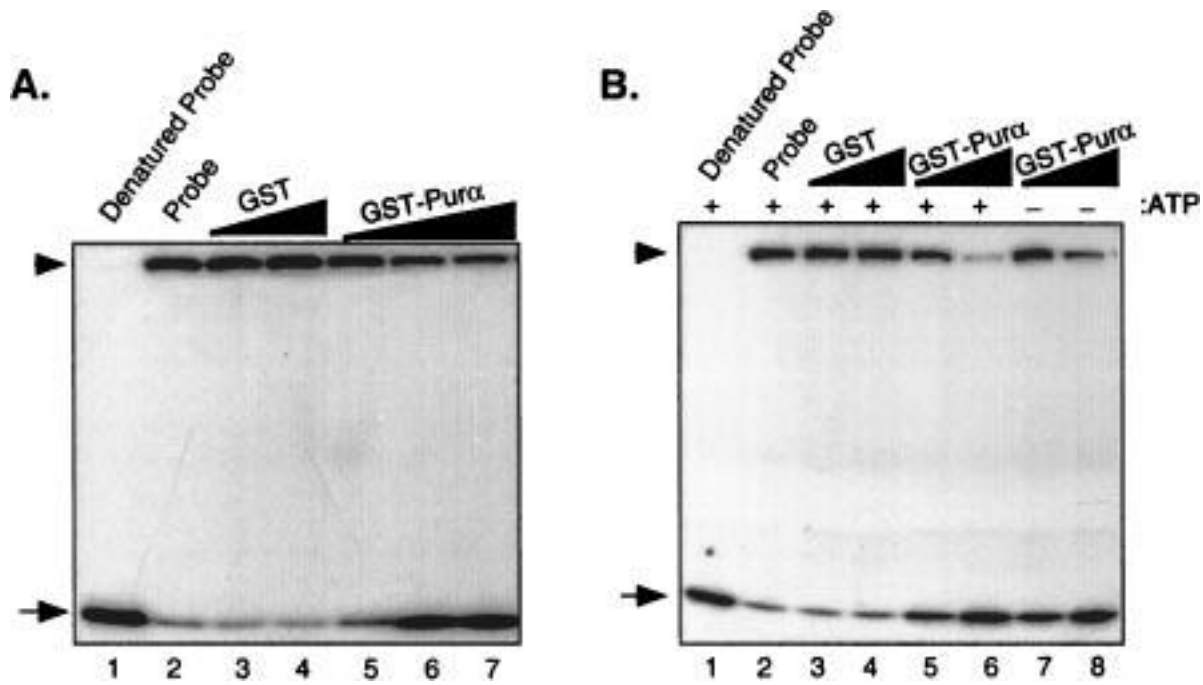

Fig. 1. DNA helix-destabilizing activity of Purα. (**A**) Helix-destabilizing assays were performed with the partially ds substrate and 50 or 500 ng of GST (**Lanes 3** and **4**) or 50, 500 or 1000 ng of GST-Purα (**Lanes 5–7**) for 60 min at 37°C. **Lane 1** contains a substrate which has been denatured at 95°C and **Lane 2** contains a substrate incubated at 37°C; both reactions were performed in the absence of either protein. (**B**) Helix-destabilizing assays were performed with the partially ds substrate and 50 or 500 ng of GST (**Lanes 3** and **4**) or GST–Purα (**Lanes 5–8**) either in the presence (**Lanes 3–6**) or the absence (**Lanes 7** and **8**) of ATP. In both panels, the arrow and arrowhead designate the displaced 18-mer and the annealed substrate, respectively.

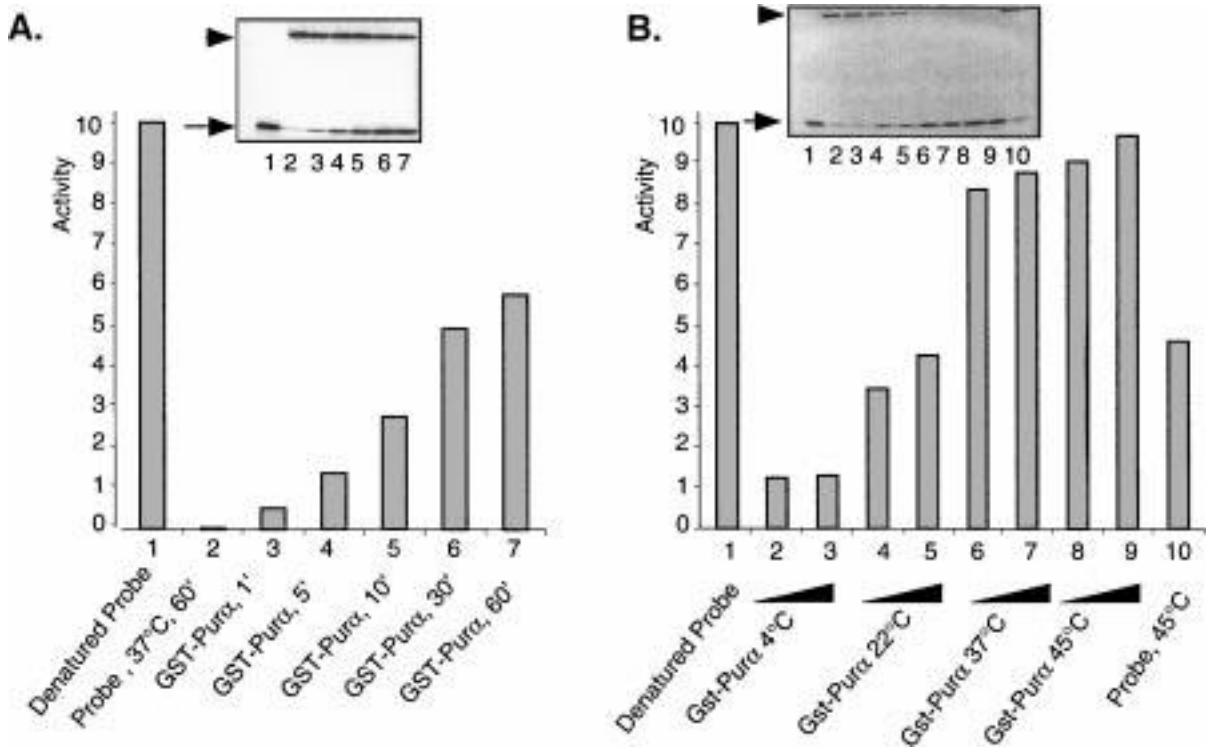

Fig. 2. Kinetics of the helix-unwinding activity of human Purα protein. (**A**) Helix-unwinding assays were performed using the partially ds substrate in the absence (**Lane 2**) or presence of 500 ng of GST–Purα for 60 min at 37°C for various times as indicated at (**Lanes 3–7**). **Lane 1** contains substrate in the absence of protein which has been denatured at 95°C. The intensity of the displaced radiolabeled 18 mer was determined by densitometry and rated in arbitrary units 1–10 with denatured DNA probe (**Lane 1**) serving as the highest point at 10. (**B**) Helix-unwinding assays were performed with the partially ds substrate and 250 and 500 ng of GST–Purα at various temperatures for 30 min. In both **A** and **B**, the arrows and arrowheads designate the displaced 18-mer and the annealed substrate, respectively. The activity was determined as described in Panel A.

stranded DNA substrate at various temperatures. As shown in Figure 2B, duplex separation was observed as the temperature of the reaction increased and reached its maximum at 45°C.

As mentioned earlier, Purα has a modular structure composed of several interesting mofits. More specifically, the central region of Purα contains three aromatic and basic repeats (designated Class I motifs) interspersed with two acidic leucine-rich repeats (designated Class II motifs) (Fig. 3, top). Previous studies have demonstrated that this region interacts with nucleic acids [Chen et al., 1995; Johnson et al., 1995]. Among the other notable structural features of Purα is the presence of an amino-terminal glycine-rich region and a glutamate-glutamine-rich domain near the carboxy terminal of the molecule. To identify the region of Purα which is responsible for the helix-destabilizing activity of this protein, a series of amino-terminal and carboxy-terminal deletion mutants were constructed and utilized in duplex-destabilizing assays. As shown in Figure 3, all amino-terminal mutants, except the smallest amino terminal mutant which contains amino acids 274–322, possess the ability to separate the labeled oligonucleotide from the M13mp18 DNA template. All of the carboxy-terminal deletion mutants, except the smallest mutant containing amino acids 1–72, also possess the ability to unwind the partially ds template. These results suggest that a large region of Purα is involved in this unwinding activity and this region spans amino acids 72–274. Interestingly, this region of Purα, which possesses DNA unwinding activity, overlaps with the DNA binding domain of Purα.

In the next series of experiments, Purα was preincubated for 30 min at various temperatures prior to the addition of duplex template DNA. As shown in Figure 4A, no significant displacement was obtained when Purα was preincubated at 95, 85, and 75°C (Lanes 2 to 4). At temperatures 65°C and lower, however, Purα exhibited unwinding activity and its

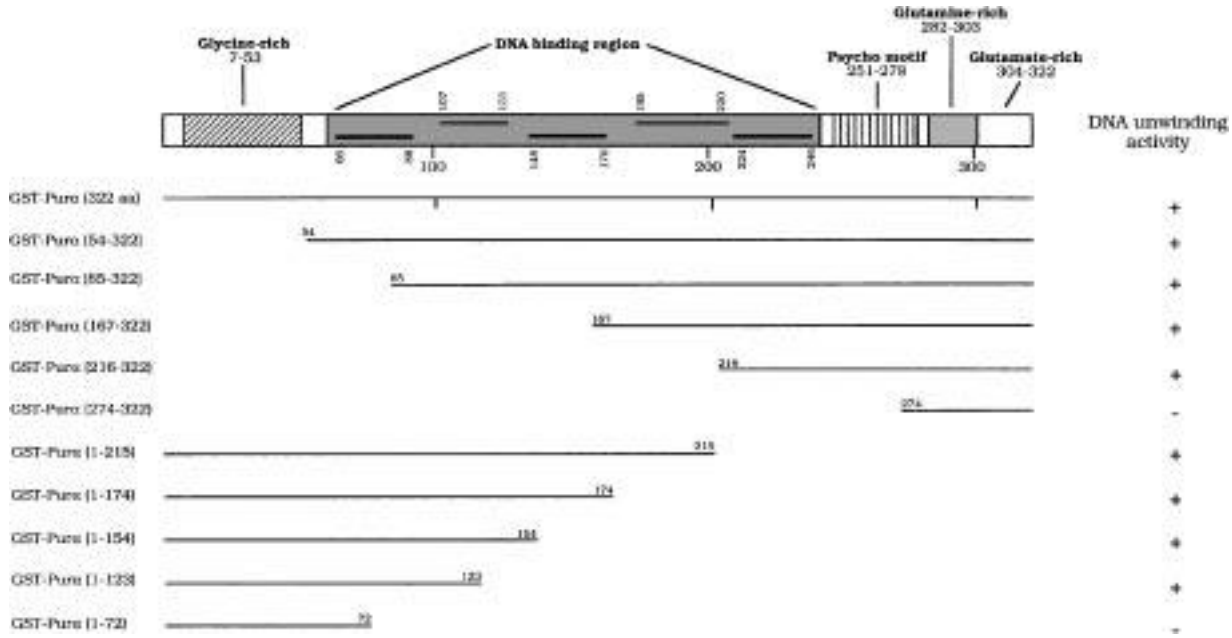

Fig. 3. Localization of the helix unwinding domain of human Purα protein. Schematic representation of human Purα protein. The three basic aromatic Class I repeats are indicated by heavy horizontal lower lines in the central repeat region and the two acidic leucine-rich Class II repeats are indicated by light horizontal upper lines in the central repeat region. The amino terminal glycine-rich region and several carboxy terminal domains are also shown. The various domains of Purα, with their respective amino acids, are indicated. Helix-unwinding assays were performed with the partially ds substrate and 500 ng of GST, GST–Purα or amino-terminal or carboxy-terminal deletion mutants of Purα for 60 min at 37°C. The helix-unwinding activity of Purα and the Purα deletion mutants are shown on the right.

maximum level was observed at 45 to 22°C (Lanes 5–9). We also performed DNA-binding electrophoretic mobility shift assays utilizing Purα which was preincubated at various temperatures and an oligonucleotide DNA probe containing the PUR binding element. As shown in Figure 4B, no complex was obtained when Purα was pre-treated at 95, 85, and 75°C (Lanes 2–4). Upon preincubation of Purα at 65°C, this protein retained its ability, albeit at a minimum level, to form a complex with the DNA probe. At lower temperatures binding activity of Purα was enhanced (compare Lanes 6–9) and maximized when Purα was pretreated at temperatures between 45 and 22°C. This pattern is reminiscent of the helix-destabilizing activity of Purα upon heat treatment (shown in Panel A). Taken together, these data demonstrate that both Purα's ability to interact with DNA and unwind DNA are severely affected upon incubation of Purα at temperatures above 65°C. This is consistent with the data presented in Figure 3 which mapped the region of Purα which is important in its DNA unwinding activity to its DNA binding domain.

Here we present evidence that Purα has the ability to unwind a partial DNA duplex and that the observed activity is ATP-independent. This is an interesting finding in light of the initial observations on the interaction of Purα with a GC/GA-rich motif (PUR element) found near the center of a region implicated as an initiation zone for chromosomal DNA replication [Vassilev et al., 1990]. This observation led us to speculate a regulatory role for Purα in the process of DNA replication. In this respect, one may speculate that Purα, by binding to the PUR element, induces unwinding at the origin of DNA replication and formation of a replication bubble. In addition, the presence of the PUR elements near the transcription start sites of several cellular [Haas et al., 1993, 1995; Thatikunta et al., 1997] and viral [Chen and Khalili, 1995; Chen et al., 1995; Chepenik et al., 1998] genes suggests a regulatory role for Purα in transcription by altering the DNA duplex structure which facilitates association of transcription factors and the synthesis of RNA. Purα also possesses a motif which consists of 18 glycine residues broken by a single

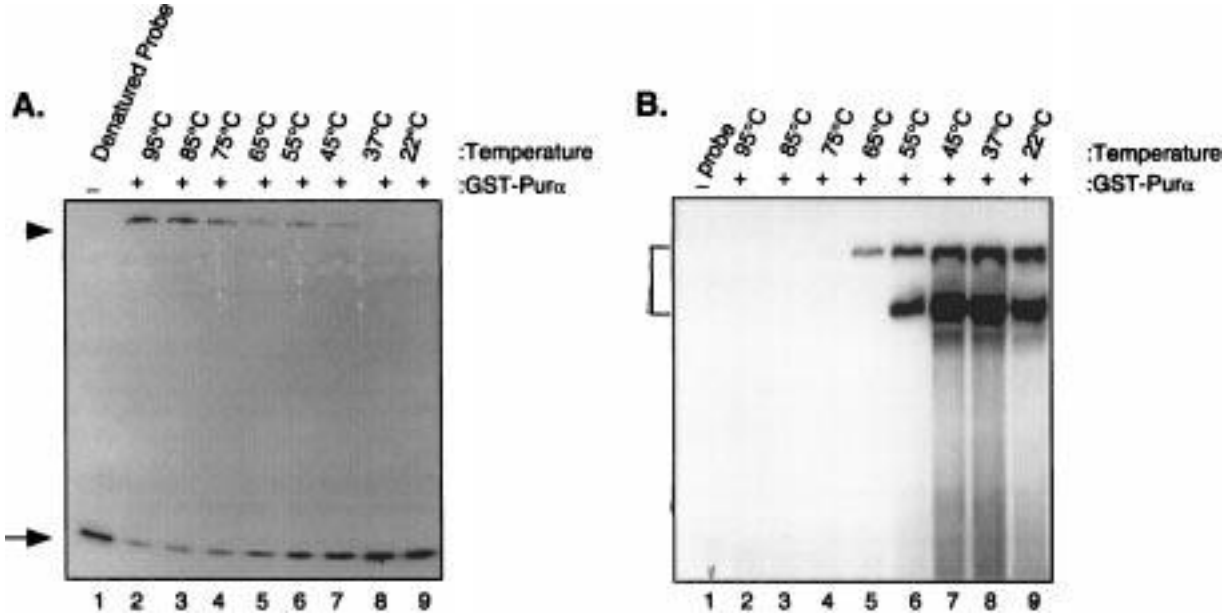

Fig. 4. Heat sensitivity of Purα on helix destabilizing and DNA binding activities. (**A**) Helix-destabilizing assay was performed with partial M13mp18 duplex DNA in the absence (**Lane 1**) or presence of 500 ng of GST–Purα which was preheated at various temperatures shown above the panel (**Lanes 2–9**) for 30 min. The arrowhead points to the position of the partial duplex DNA and the arrow depicts the displaced [^{32}P]-labeled oligonucleotide. (**B**) Band-shift assay was performed in a reaction containing 500 ng of preheated GST–Purα as described above and [^{32}P]-labeled oligonucleotide containing the PUR element. The positions of the DNA–protein complexes (mono- and multimer Purα) are shown by the bracket.

serine residue. Similar glycine stretches are found in proteins with helix-destabilizing activities [Haynes et al., 1987]. In many respects, Purα is functionally similar to SV40 T-antigen, a well-studied regulatory protein which controls replication and transcription of the viral genome. This protein interacts with the region within and adjacent to the viral core replication origin which is enriched in G and C residues. Further, T-antigen has unwinding and helicase activities and unlike Purα, these events require hydrolysis of ATP [Pipas 1992]. Interestingly, earlier results indicated that human polyomavirus T-antigen, which has significant homology to SV40 T-antigen, interacts with Purα [Gallia et al., 1998]. While the importance of this interaction in the unwinding and helicase activities of Purα and T-antigen remains to be investigated, results from functional studies revealed that overexpression of Purα inhibits the stimulatory activities of T-antigen on transcription and replication [Chang and Khalili, 1995; Gallia et al., 1998]. Studies are currently in progress to examine the cooperativity between Purα and JCV T-antigen and various cellular proteins which are involved in replication of DNA in eukaryotic cells.

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