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Purification and characterisation of dRP-A: a single-stranded DNA binding protein from *Drosophila melanogaster*

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Abstract

Replication protein A (RP-A) is an essential single-stranded DNA binding protein (SSB) involved in the initiation and elongation phases of eukaryotic DNA replication. It has the ability to bind single-stranded DNA extremely tightly and possesses a characteristic hetero-trimeric structure. Here we present a method for the purification of RP-A from *Drosophila melanogaster* embryos. *Drosophila* RP-A (dRP-A) has subunits of about 66, 31 and 8 kDa, in line with analogues from other species. It binds single-stranded DNA very tightly via the large subunit. The complete protein has at least a 10- to 20-fold preference for single-stranded DNA over double-stranded DNA and it appears that binding is only weakly co-operative. Band shift experiments suggest that it has an approximate site covering the size of 16 nucleotides or less, however, it shows a greater affinity for long oligonucleotides than for short ones. We also demonstrate that dRP-A can stimulate the activity of its homologous DNA polymerase α in excess of 20 fold. Analysis of the protein's abundance during embryo development indicates that it varies in a manner akin to other replication proteins.

Key words: Single-stranded DNA binding protein; RP-A; DNA replication; *Drosophila melanogaster*

1. Introduction

Eukaryotic single stranded binding protein (SSB), also called RP-A (replication protein A) and RF-A (replication factor A), was first identified as an important replication component during fractionation of an in vitro system for the replication of simian virus 40 (SV40) [1–3]. Since its isolation from human cell extracts, a number of analogues have been found in various systems, including *Saccharomyces cerevisiae* [4,5], calf thymus [6], the trypanosomatid *Crithidia fasciculata* [7], and *Xenopus laevis* [8]. RP-A appears to function in several aspects of DNA metabolism: in vitro studies have implicated the protein not only in DNA replication (both initiation and elongation), but also DNA repair [9] and DNA strand exchange reactions [10]. Its involvement in replication has been further confirmed by genetic studies in yeast [5].

For all organisms so far investigated, the structure of RP-A is well conserved, namely a heterotrimer consisting of tightly bound polypeptides, with molecular masses of approximately 70 kDa, 34 kDa and 12 kDa, (an exception to this is in the trypanosomatid where the large subunit is only 51 kDa). The individual functions of these three subunits are still not fully elucidated. The best understood is the 70 kDa polypeptide which contains the DNA binding domain [4]. Neither of the other subunits bind significant amounts of DNA, and in fact appear to be completely dispensable for DNA binding by the 70 kDa subunit [11]. However, the same study demonstrated that at least one of these subunits must carry out an important function since the 70 kDa subunit alone cannot substitute efficiently for the complete RP-A complex in SV40 DNA replication. There is at present little evidence concerning the functions of the smaller subunits, although it has been speculated that they may be involved in the various protein interactions observed for RP-A [12]. Perhaps relevant to this is the observation that the phosphorylation state of the 34 kDa peptide can vary [4] and, at least for the human protein, these variations seem to correlate with specific stages of the cell cycle [13,14]. Although this might imply some role for this polypeptide in control, the mechanism and significance of this modification have yet to be determined.

Biochemical characterisation of RP-A has concentrated on two aspects of its activity. The first of these is its interaction with single-stranded (ss) DNA. The affinity of the protein for ssDNA is very high: in the region of 10^9–10^{10} M^{-1} [10,15]. For all homologues studied, double-stranded (ds) DNA binding is weak compared with that of ssDNA, however, reports of the relative affinities range from a 10-fold [1] to a 1,000-fold preference [4,16]. There is also some degree of variation in the reported values for binding site size, (90–100 [10] vs. 20–30 nucleotides per RP-A heterotrimer [6,15]) and also

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Abbreviations: ss, single stranded; ds, double stranded; SSB, single-stranded DNA binding protein: RP-A, replication protein A; SV40, simian virus 40; T-ag, SV40 large tumour antigen.

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the co-operativity of binding from high [10], through weak [6], to low or none [15].

The other area of recently increased interest are the interactions that RP-A has with other proteins. ssDNA binding is probably the main function of RP-A during initiation, as any SSB (including *E. coli* SSB) can efficiently stimulate SV40 large T antigen (T-ag)-mediated unwinding of the origin region of SV40 [17]. However, the complete SV40 in vitro replication system shows species specificity with respect to RP-A. This is most likely due to a different property of the enzyme: its capacity to stimulate the activity of the replicative polymerases α [7,11,18], δ [18] and ε [19] under certain conditions. The best studied of these is the stimulation of the DNA polymerase α where stimulation is quite specific. Efficient stimulation is mainly achieved if the polymerase and the RP-A are isolated from the same species, although non-homologous RP-As have been reported to cause stimulation at reduced levels [7,17,20]. As is the case for DNA binding, the 70 kDa subunit alone is sufficient to give stimulation [11]. A recent study [21] suggests that this interaction is with the primase subunit (the 48 and/or 58 kDa subunits) of DNA polymerase α, while another report [6] indicates that RP-A interacts with the main catalytic subunit (180 kDa). An additional protein interaction of human RP-A with the T-ag of SV40 has also been reported [17].

Using a rapid procedure we have been able to purify RP-A from *Drosophila melanogaster* (dRP-A) to near homogeneity. This procedure is reported here together with a characterisation of the protein with respect to its structure, covering size, affinity for ssDNA and its effect on its cognate DNA polymerase α. In addition we present evidence for the abundance of dRP-A during development. These results demonstrate that dRP-A shows strong similarities to RP-A from other species.

2. Materials and methods

2.1. Reagents

General laboratory chemicals were purchased from Sigma (Poole, Dorset), as was ssDNA cellulose. Radionucleotides were from Amersham (Amersham, Bucks). DNase I (RNase free) was from Boehringer-Mannheim (Lewes, E. Sussex). T4 polynucleotide kinase was from Northumbria Biologicals Ltd. (Cramlington, Northumberland). Alkaline phosphatase-conjugated goat anti-rabbit antibody was supplied by Jackson Immunoresearch (West Grove, PA). Acrylamide monomer (Protogel) was purchased from National Diagnostics (Aylesbury, Bucks.).

2.2. DNA

Oligonucleotides (17, 25, 32, 38 and 59 nucleotides in length) were synthesized on a Pharmacia Gene Assembler Plus. Their sequences were: 17mer, 5′-GTAAAACGACGGCCAGT; 25mer, 5′-GGCCCGTCTTCCGGTGCTTCAATCG; 32mer, 5′-GGCCTCCTACAATTGAAGCCAGCAGTTTTCGC; 35mer, 5′-TATCGTAGCTCTGTTATCGATATGTTCTCGGCATT; 38mer, 5′-GCCAAGCTTGGCTGCAGGTCGACGGATCCCCGGGAATT; 59mer, 5′-AACAGCTATGACCATGAACAAAATAGATCGATCTAAACTATGACTGGCCGTCGTTTTAC.

The ds oligonucleotide required for one experiment was prepared by annealing the 35mer to its compliment in equimolar propertions.

2.3. Protein assays

These were carried out using the method of Bradford [22]. Protein assay solution was purchased from Bio-Rad.

2.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

This was conducted according to the method of Laemmli [23].

2.5. Purification of Drosophila RP-A (dRP-A)

A typical preparation was from 60 g of 0–6 h *Drosophila melanogaster* embryos (wild-type, Oregon R). The purification was basically according to the method of Brown et al. [7] with some modifications. The embryos were homogenised in a dounce homogeniser (using both the B and then the A pestles in series) in 150 ml of homogenisation buffer (50 mM HEPES (pH 7.5), 10 mM EDTA, 0.5 M NaCl, 2.5 mM 2-mercaptoethanol, 2 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 mM sodium metabisulphite and 10 mM benzamidine). After homogenisation the lysate was stirred for 40 min at 4°C and then centrifuged at 42,000 rpm in a Beckman Ti45 rotor for 30 min at 3°C. The middle layer (of three) was carefully removed using a Pasteur pipette and re-centrifuged under the same conditions but for 60 min. Again the middle layer was removed and passed through a double layer of Miracloth (from Calbiochem, La Jolla, CA). The filtered lysate was centrifuged in a Beckman Ti45 one further time (42,000 rpm for 30 min at 3°C) in order to remove contaminating lipid (top layer). The lower layer (at this stage there was no pellet) was loaded onto a 2.5 × 2.5 cm ssDNA cellulose column equilibrated with 25 mM HEPES (pH 7.5), 10% (v/v) glycerol, 1 mM EDTA, 0.02% (w/v) Brij 58, 2.5 mM 2-mercaptoethanol, 1 μg/ml leupeptin, 0.5 μg/ml pepstatin A, 0.25 mM PMSF and 5 mM sodium metabisulphite (buffer A) containing 0.5 M NaCl. The column was washed sequentially with 4 vols. of buffer A containing 0.5 M NaCl and 3 vols. of buffer A containing 0.75 M NaCl. Protein was eluted with buffer A containing 1.5 M NaCl and 50% (v/v) ethylene glycol. Fractions that contained protein were pooled and dialysed, against 25 mM imidazole hydrochloride (pH 7.5), 10% (v/v) glycerol, 0.1 mM EDTA, 0.02% Brij 58, 2.5 mM 2-mercaptoethanol, 1 μg/ml leupeptin, 0.5 μg/ml pepstatin A, 0.25 mM PMSF and 5 mM sodium metabisulphite (buffer B) containing 50 mM NaCl. Precipitated material was removed by centrifugation of the dialysate in a bench-top centrifuge. The supernatant was passed through a 0.2 μm nitrocellulose filter before being loaded onto a 1 ml Mono Q column (HR 5/5 from Pharmacia) which had been equilibrated with buffer B containing 50 mM NaCl. The column was washed with 10 ml buffer B containing 50 mM NaCl at which point a 20 ml linear gradient was applied from 50 to 400 mM NaCl in buffer B. Fractions of 0.5 ml were collected and analysed.

2.6. Production of antiserum

Antibody to RP-A was generated by immunisation of a rabbit with the purified protein.

2.7. Labelling of oligonucleotides

These were labelled at the 5′ end as described in Sambrook et al. [24] with the modification that only 5 pmol of DNA was labelled at a time.

2.8. Labelling of dsDNA

A 230 bp fragment of the common region of the tomato golden mosaic virus (TGMV) was labelled as described in Thömmes et al. [25] by means of the polymerase chain reaction (PCR). ^{32}P was incorporated by using [^{32}P]dCTP; this same DNA was made amenable to UV cross-linking to protein by incorporation of bromo-dUTP in place of dTTP in the reaction. To produce ssDNA substrate the DNA was heated to 100°C for 5 min and then cooled rapidly in an ice bath.

2.9. Gel retardation experiments

The indicated amount of DNA (always ss except in the case of the ss/ds competition experiment) and dRP-A were incubated together in a total volume of 20 μl FB buffer (1 × FB is 30 mM HEPES (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, 0.5% inositol, 1 mM dithiothreitol). The reactions were incubated for 25 min at room temperature after which they were brought to a final concentration of 5% glycerol (v/v), 0.004% (w/v) Bromophenol blue and 0.004% (w/v) xylene cyanol FF. They were then loaded onto polyacrylamide gels of an appropriate acrylamide concentration (from 8 to 12%) depending on the length of DNA being used. These were run in 1 × TBE (89 mM Tris-borate, 89 mM boric acid and 2 mM EDTA) at 20 V/cm for 3 h at 4°C. These gels were fixed in

10% methanol/10% acetic acid and then dried onto Whatman DE81 (DEAE-cellulose paper) prior to autoradiography.

2.10. UV cross-linking experiments

1.25 ng of the 230 bp DNA (made ss by heat denaturation) was incubated with 0.8 μg of dRP-A, in a total volume of 20 μl, at 0°C in the presence of buffer B containing 50 mM NaCl. After 30 min the reactions were subjected to 2 J of UV radiation, using a Stratalinker (Stratagene). Reactions were adjusted to a final concentration of 10 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT and 0.1 mg/ml BSA and incubated with 10 U of DNase I for 30 min at 37°C. These mixtures were then boiled in the presence of SDS-PAGE gel loading dye and loaded onto a 12% polyacrylamide gel. Proteins were visualised by staining with Coomassie blue R250, before drying onto Whatman 3MM paper for autoradiography.

2.11. Western blotting

SDS-PAGE gels were run as described above. Afterwards the proteins were electro-transferred to nitrocellulose filters for 60 min at 2 mA per cm^2 using a Bio-Rad electro-transfer cell. Blots were blocked in 5% (w/v) low fat milk powder, 1 × Tris-buffered saline (TBS; 10 mM Tris-HCl (pH 8), 150 mM NaCl) and 0.01% (v/v) Tween 20 for at least 45 min. Rabbit anti-dRP-A antiserum was incubated with the blot at a dilution of 1 in 1,000 in the presence of 1% (w/v) low fat milk powder, 1 × TBS and 0.01% (v/v) Tween 20 for at least 60 min. The blot was washed 3 × 10 min with 1 × TBS and then incubated with alkaline phosphatase-conjugated, goat anti-rabbit IgG antibody at a dilution of 1 in 7,500, again in 1% (w/v) low fat milk powder, 1 × TBS and 0.01% (v/v) Tween 20 for at least 60 min. The blot was once more washed for 3 × 10 min in 1 × TBS and the bands were visualised by incubation in 20 ml of AP buffer (100 mM Tris-HCl (pH 9.5), 100 mM NaCl and 5 mM $MgCl_2$) with 132 μl Nitroblue tetrazolium (50 mg/ml in 70% dimethyl formamide) and 66 μl 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml in dimethyl formamide).

2.12 DNA polymerase α assays

These were based on the methods of Kenny et al. [18]. Generally, 30 μl reaction mixtures contained 30 mM HEPES (pH 7.5), 7 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.12 μg of poly dA · $(dT)_{12}$ (20:1, w/w), 2.2 μM [^{3}H]dTTP and 0.06 U of *Drosophila* DNA polymerase α. After incubation at 37°C, TCA-precipitable radioactivity was determined by liquid scintillation counting. In the case of time–course experiments, 0.2 μg of poly dA · $(dT)_{12}$ (20:1 w/w) was used in 50 μl reactions from which 8 μl aliquots were removed at intervals and assayed.

3. Results

3.1. Purification of RP-A from Drosophila melanogaster

We have purified the RP-A from *Drosophila melanogaster* embryos by the use of a ssDNA–cellulose column followed by Mono Q column chromatography. As for other RP-As, dRP-A remains bound to ssDNA even at 0.75 M NaCl, allowing the vast majority of proteins to wash through, before elution with 1.5 M NaCl containing 50% (v/v) ethylene glycol. The dRP-A eluted from the FPLC Mono Q column between ~160 mM and ~205 mM NaCl. Fig. 1 shows an SDS polyacrylamide gel of the column peak fraction; it demonstrates the usual subunit structure of the eukaryotic RP-As, namely 3 polypeptides of M_r ~66 kDa, ~31 kDa and ~8 kDa (not always easily seen). In some preparations an additional band is sometimes apparent at 45 kDa. This is almost certainly a break-down product of the 66 kDa subunit, similar to the 55 kDa band observed for calf thymus RP-A [6] and the 53 kDa band seen in some human RP-A preparations [2,15]. From silver-stained gels we would estimate that, at this stage, the protein is at least 95% pure. As with the RP-As from other organisms a doublet can sometimes be observed at 31 kDa, however, most of our preparations mainly contain the unphosphorylated form.

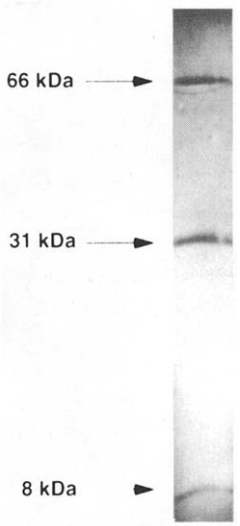

Fig. 1. SDS PAGE analysis of dRP-A. 0.5 μg of dRP-A was separated on a 15% SDS-polyacrylamide gel and stained with silver.

3.2. The abundance of Drosophila RP-A varies during development

This was investigated by Western blot analysis of protein extracts made from various developmental stages of

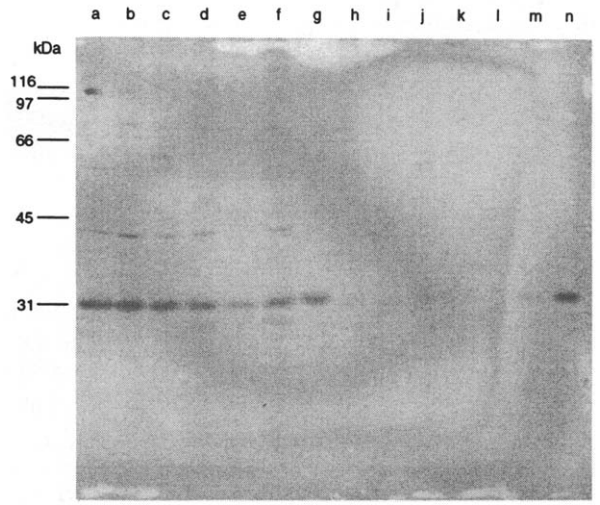

Fig. 2. Variation in levels of dRP-A during *Drosophila* development as analysed by immunoblot. Equivalent protein extracts from various stages during *Drosophila* development were separated on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with anti-dRP-A polyclonal antiserum. Lanes a–f are embryonic stages: a, 0–4 h; b, 4–8 h; c, 8–12 h; d, 12–16 h; e, 16–20 h; f, 20–24 h; g, h and i are 1st, 2nd and 3rd instar, respectively; j, early pupa; k, late pupa; l, adult male; m, adult female; and n, 0.5 μg of purified dRP-A. Protein standards with molecular weights indicated on the left of each panel were: myosin (200 kDa); β-galactosidase (116 kDa); phosphorylase B (97 kDa); BSA (66 kDa); ovalbumin (45 kDa); carbonic anhydrase (31 kDa); trypsin inhibitor (21 kDa); lysozyme (14 kDa); aprotinin (6 kDa).

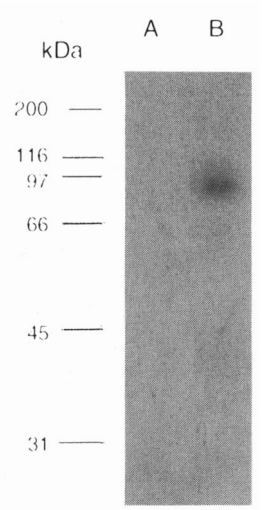

Fig. 3. Binding of the dRP-A subunits to DNA. Protein from the peak fraction of the Mono Q column was cross-linked to ^{32}P-labelled ssDNA. After digestion of unbound DNA with DNase I the labelled polypeptides were analysed by SDS-PAGE on a 12% gel followed by autoradiography. (A) No protein, (B) 0.8 μg of dRP-A. Numbers at the left indicate the size of protein standards as in Fig. 2.

Drosophila. The lanes were loaded with equal amounts of total protein. The antiserum used was raised against the complete dRP-A but is most active against the 31 kDa subunit. Fig. 2 demonstrates that the amount of dRP-A is high in the early embryonic stages, and then declines during development until there is a resurgence of abundance in the first instar larvae. In addition, protein could be observed in adult females but not in adult males. This profile is consistent with that of a protein involved in replication, since it is detected in those stages when replication is proceeding fastest. No change in the relative abundance of the phosphorylated vs. the unphosphorylated form of the 31 kDa subunit could be seen during development.

3.3. Identification of the ssDNA binding subunit

In all RP-As so far characterised this function has been attributed to the large subunit. Here, identification was carried out by UV cross-linking ^{32}P-labelled DNA to dRP-A and running the products out on a 12% SDS-polyacrylamide gel. Fig. 3 shows an autoradiograph of such a gel; it shows that ssDNA is cross-linked to the large (66 kDa) subunit. The band has an apparent size slightly greater than expected due to the additional mass attributable to the cross-linked DNA. Fig. 3 also shows the same experiment carried out in the absence of RP-A; it indicates that the band seen is due to RP-A and does not arise from the labelled DNA itself.

3.4. dRP-A has a preference for ssDNA over dsDNA

In order to assess the relative affinities of dRP-A for ss and dsDNA, dRP-A and labelled ssDNA were incubated together with increasing amounts of unlabelled competitors in a bandshift assay. As can be seen in Fig. 4, the effect of 50- and 100-fold excess dsDNA is almost negligible (lanes c and d, respectively) when compared with the control (lacking competitor, lane b), while lanes e and f show that the same molar amounts of ssDNA diminishes the shifted band significantly. We would estimate that dRP-A has a preference for ssDNA over dsDNA of at least 10-fold.

3.5. Study of site size and co-operativity

This was achieved by means of gel retardation assays with various lengths of ssDNA. Oligonucleotides of 17, 25, 32, 38 and 59 bases in length were synthesised chemically; a 230 bp fragment was produced by PCR followed by heat denaturation (see section 2). Fig. 5 shows gel retardation assays demonstrating the effect of adding increased amounts of RP-A to the DNA fragments of different length. From this it can be seen that for DNA of 17 and 25 nucleotides in length (A and B) only a single retarded band occurs. This is consistent with only one dRP-A heterotrimer bound to DNA of such lengths, even at high protein concentrations. In the panels displaying retardation of DNA of 32 and 38 nucleotides (C and D), two bands of lower mobility can clearly be observed, the slowest band only being observed at higher protein concentrations and apparently arising after the first band. These low mobility bands most likely indicate two molecules of dRP-A binding to the DNA. These observations suggest that the binding of one molecule of dRP-A may require 16 nucleotides or less. Further evidence of a small-sized binding site comes from panel E which shows the gel retardation observed for DNA of 59 nucleotides in length. This clearly shows three discrete bands, indicating a loading of up to three dRP-A mole-

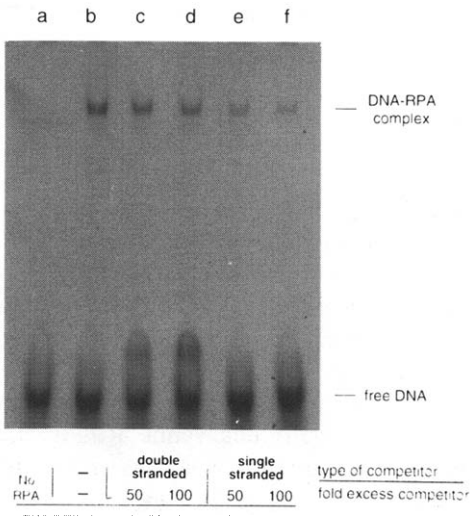

Fig. 4. Competitive bandshifting assay to assess the affinity of dRP-A for ss or dsDNA. 15 nM dRP-A was incubated together with 0.2 nM of a labelled 35mer and indicated amounts of unlabelled ss or ds competitor DNA. The complexes formed were analysed by native gel electrophoresis followed by autoradiography. Lane a, labelled DNA only (i.e. no dRP-A); lane b, no competitor; lanes c and d, 50- and 100-fold excess of dsDNA; lanes e and f, 50- and 100-fold excess of ssDNA.

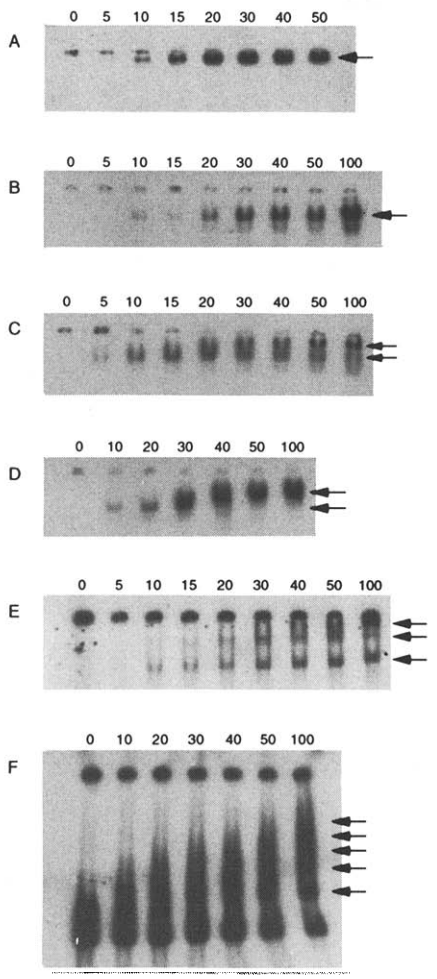

Fig. 5. Interaction of dRP-A with various lengths of DNA as measured by gel retardation assays. Increasing amounts of dRP-A (nM, as indicated on top of each lane) were incubated with labelled ssDNAs of various length. Analysis of the complexes formed was by native gel electrophoresis followed by autoradiography. (A) 17mer oligonucleotide at 0.2 nM; (B) 25mer oligonucleotide at 0.1 nM; (C) 32mer oligonucleotide at 0.1 nM; (D) 38mer oligonucleotide at 0.4 nM; (E) 59mer oligonucleotide at 0.1 nM; (F) 230 nucleotide heat-denatured PCR product at 0.66 nM. Arrows indicate the positions of retarded bands.

cules per 59 bases. The final panel of Fig. 5 (230 nucleotides) shows that with still longer DNA, multiple bands are visible and a progression to slower migrating species correlates with an increase in protein concentration. It also provides tentative evidence of a lack of strong co-operativity of dRP-A binding to ssDNA. If co-operativity was high, there would be a different distribution in the appearance of the shifted bands, with a tendency to bind preferentially to DNA which is already associated with protein.

3.6. *DNA binding affinity varies with ssDNA size*

The relative affinity of dRP-A for DNA of different lengths was determined using a competitive band shift assay. The efficiency with which oligonucleotides of different sizes competed for binding to a 32mer was taken to be a measure of the relative affinity of the dRP-A for various sizes of DNA. As can be seen from Fig. 6, the affinity of RP-A for DNA increases as the length of the oligonucleotide increases, at least up to lengths of 59 nucleotides. The relative binding affinity of a 59mer to a 17mer is about 50:1.

3.7. *dRP-A stimulates its homologous DNA polymerase α*

Fig. 7A shows that dRP-A is a very potent activator of its cognate DNA polymerase α and that the stimulation observed is in excess of 20 times. Fig. 7B demonstrates that the stimulation occurs over the whole time course of the reaction. The same stimulation cannot be achieved using *E. coli* SSB (data not shown), suggesting the importance of specific protein–protein interactions, rather than just an effect of a removal of secondary structures in the ssDNA substrate.

4. Discussion

The data presented in this paper suggest that the RP-A we have purified from *Drosophila* is very similar to that already isolated from several other higher eukaryotes. It is a heterotrimer consisting of subunits of approximately 66, 31 and 8 kDa, which shows a marked preference for binding to ssDNA vs. dsDNA (> 10-fold). It is also capable of stimulating its cognate DNA polymerase α by a factor of at least 20. These observations are in complete agreement with data reported previously on dRP-A [26]. We have further shown that its pattern of expression in the developing embryo is consistent with its involvement

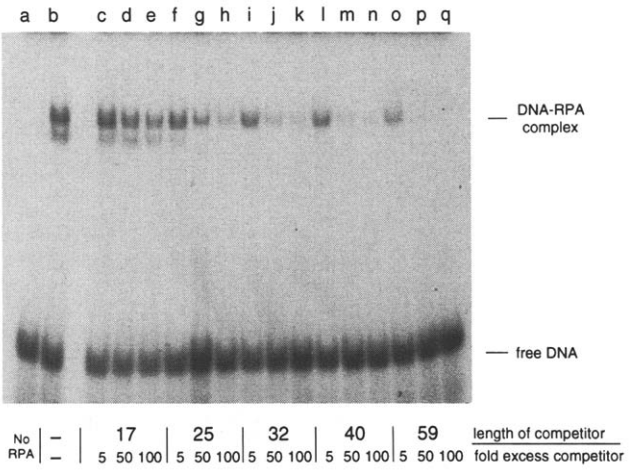

Fig. 6. Competitive bandshifting assay to assess the affinity of dRP-A for ssDNA of different sizes. 15 nM dRP-A was incubated together with 0.2 nM of a labelled 32mer and various amounts of unlabelled competitor DNA. The complexes formed were analysed by native gel electrophoresis followed by autoradiography. Lane 1, labelled DNA only (i.e. no dRP-A); lane 2, no competitor; lanes 3–17, competition experiments with (lanes 3–5) 17mer, (lanes 6–8) 25mer, (lanes 9–11) 32mer, (lanes 12–14) 38mer, and (lanes 15–17) 59mer. In each case the competing oligonucleotide was in 5 ×, 50 × and 100 × molar excess.

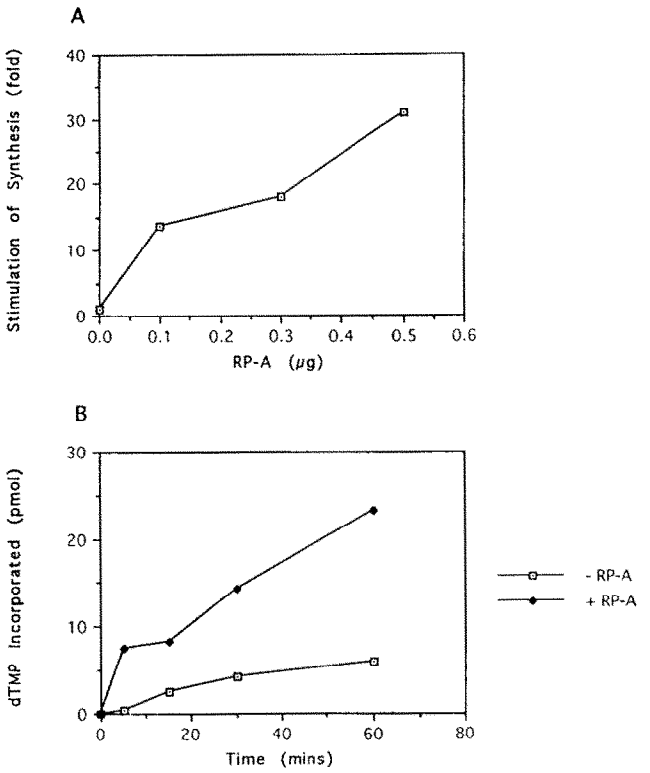

Fig. 7. dRP-A stimulates ist cognate DNA polymerase α. (A) Effect of increasing concentrations of dRP-A: 0.06 U *Drosophila* DNA polymerase α were incubated with 0.12 μg poly [dA]:dT[12] and an amount of dRP-A as indicated. Polymerisation was assayed as described in section 2. Stimulation of synthesis was calculated relative to DNA polymerase activity in the absence of RP-A. (B) Time–course of stimulation of *Drosophila* DNA polymerase α by dRP-A: 0.06 U *Drosophila* DNA polymerase α were incubated with 0.2 μg poly [dA] · dT[12] and 0.4 μg dRP-A. Polymerisation was assayed as described in section 2.

in DNA replication. In addition the largest subunit (66 kDa) appears to be responsible for binding to DNA. The affinity for ssDNA we have calculated is comparable with that for other RP-As [10,15]. As far as the site size and the co-operativity measurements are concerned, our data suggest a small site binding size and very little co-operativity. The binding characteristics of dRP-A to oligonucleotides of 17, 32 and 59 bases suggest that the length of DNA required for stable interaction may be 16 nucleotides or less. This is smaller than the previous measurements for dRP-A and also other organisms [26]. While we cannot rule out the possibility that the double band shift in the 32mer actually represents partial site coverage of the second molecule of dRP-A we feel that this is less likely since there is little difference in the protein concentration dependence of the second band in the 32mer vs. the 38mer. Similar double binding to DNA as short as 36 nucleotides has also been reported recently [27]. We therefore feel that the differences observed are likely to be due to differences in the method of measurement [26] or the experimental conditions [10]. In this respect the eukaryotic RP-As may share features with *E. coli* SSB which shows variability in its binding characteristics with changes in the solution conditions [28,29].

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