Volume 308, number 3, 240–244 FEBS 11436 August 1992


# DNA unwinding activity of replication protein A

Anthi Georgaki, Bettina Strack, Vladimir Podust and Ulrich Hübscher

*Department of Pharmacology and Biochemistry, University of Zürich-Irchel, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland*

Received 1 July 1992

Replication protein A (RP-A) is a heterotrimeric complex conserved in eukaryotic cells. It binds to single-stranded DNA and is essential for initiation and elongation of DNA replication. In this communication we give evidence that this protein can unwind DNA independent of magnesium and ATP, two essential cofactors for *bona fide* DNA helicase activity. RP-A can unwind up to at least 350 basepairs and appears to be required in stoichiometric amounts. The reaction is extremely sensitive to NaCl and $MgCl_2$. This activity of RF-A is suggestive for a possible unwinding function in initiation of DNA replication in eukaryotes.

Replication protein A; DNA unwinding; Initiation; DNA replication

## 1. INTRODUCTION

Replication protein A (RP-A, also called replication factor A or eukaryotic single-stranded binding protein) is a heterotrimeric protein that is highly conserved in eukaryotic cells (see e.g. [1] and citations therein). The complex is composed of three polypeptides with molecular weights of 70k, 32k and 11k, respectively. All three proteins are essential for the viability of *Saccharomyces cerevisiae* [2–4], and important roles of RP-A have been proposed in the initiation events of eukaryotic DNA replication [5]. RP-A interacts with Simian virus 40 large T antigen and with DNA polymerase α/primase [6,7]. The 70 kDa subunit binds to single-stranded DNA, one RP-A molecule covering about 30 bases and preferring pyrimidine bases for its binding [1]. Furthermore, the 32 kDa subunit is phosphorylated in a cell cycle-dependent manner [8]; this phosphorylation appears to occur with the replication initiation complex [9] and activation of DNA replication by RP-A appears to be performed by the cdc2 family kinases [10]. In addition, interaction with DNA polymerase δ [11], and cellular DNA helicases [12] have been described. Finally, RP-A has been identified very recently in nuclear preinitiantion centers poised for DNA synthesis in *Xenopus laevis* egg extracts [13]. In summary, RP-A appears to have a central function in the DNA replication complex by virtue of its interaction with several parts of the replication machinery.

*abbreviations:* RP-A, replication protein A; RF-C, replication factor C; PCNA, proliferating cell nuclear antigen; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gelelectrophoresis.

*Correspondence address:* Dr. U. Hübscher, Department of Pharmacology and Biochemistry, University of Zürich-Irchel, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland. Fax: (41) (1) 362 0501.

In this report we demonstrate that in the absence of salt RP-A can unwind up to at least 350 basepairs of DNA, and that this unwinding activity is independent of $MgCl_2$ and ATP, two essential cofactors for *bona fide* DNA helicases.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Nucleotides, column supports, nucleic acids and other chemicals were exactly as described [12]. Bacteriophage gene 32 protein was from Pharmacia, *Escherichia coli* single-stranded binding protein was puri-

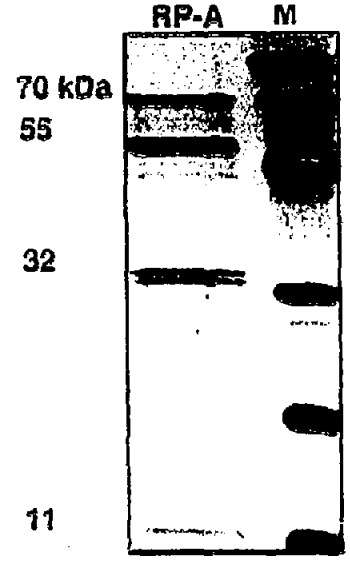


Fig. 1. SDS-PAGE of calf thymus RP-A. RP-A was purified as described in Materials and Methods and 13.5 μg electrophoresed in 12.5% SDS-PAGE. The gel was stained with Coomassie blue. Markers were: rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), hen eggwhite ovalbumin (42.7 kDa), bovine carbonic anhydrase (31 kDa) soybean trypsin inhibitor (21.5 kDa) and hen egg white lysozyme (14.4 kDa).


*Published by Elsevier Science Publishers B.V.*

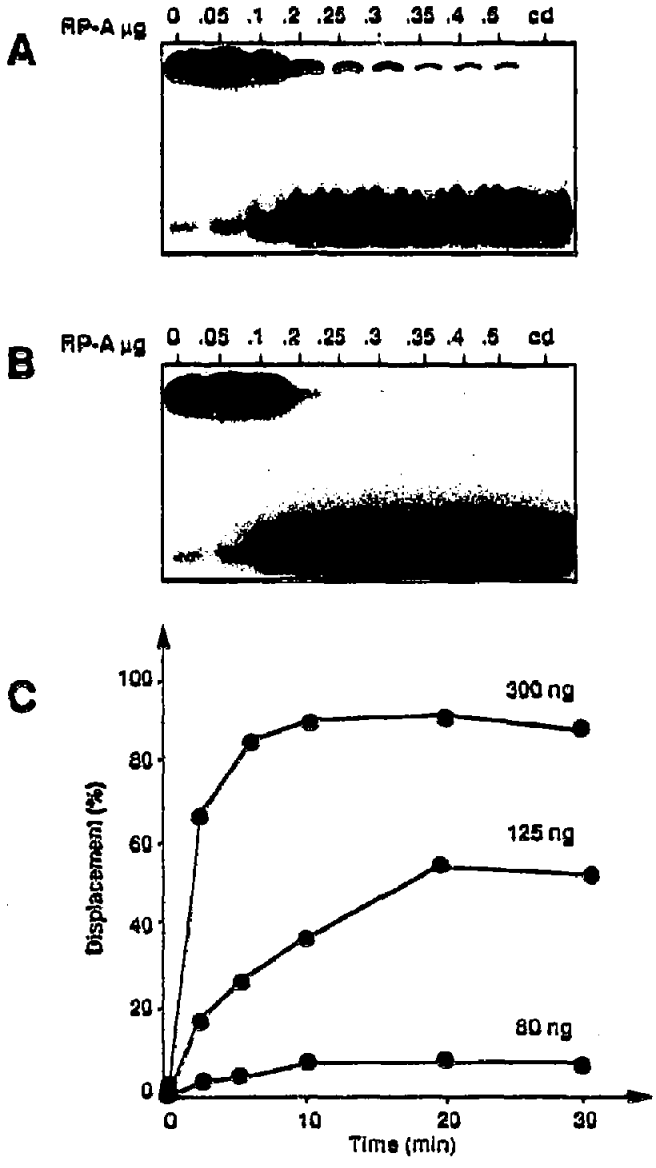


Fig. 2. DNA unwinding by calf thymus RP-A on the short (24mer) substrate. DNA unwinding was performed as described in Materials and Methods. (A) Titration of RP-A in the presence of $MgCl_2$ (1 mM) and ATP (1 mM). (B) Titration of RP-A in the absence of $MgCl_2$ and ATP. (C) Time course of unwinding with the indicated amounts of RP-A per time point. cd = substrate boiled for 3 min.

fied from the overproducer RLM 727 (gift from M. Defais, CNRS, Toulouse), and the adenovirus DNA binding protein donated by P.C. van der Vliet (Utrecht).

2.2. *Assay for RP-A*

The fate of RP-A during isolation was followed with two assay: (i) by testing individual fractions with a polyclonal antibody raised in chicken; and (ii) by in vitro complementation of DNA polymerase δ, proliferating cell nuclear antigen (PCNA) and replication factor C (RF-C) on a singly-DNA primed M13 DNA template [14].

2.3. *Purification of RP-A*

*Buffer A:* 20 mM Tris-HCl (pH 8.0), 10 mM KCl, 250 mM sucrose, 10% (v/v) glycerol, 1 mM dithiothreitol, 2 mM EDTA, 0.5 mM spermidin, 10 mM benzamidine, 10 mM sodiumbisulfite, 1 mM PMSF, 0.5 mg/ml TLCK and 1 µg/ml each of pepstatin, leupeptin, aprotinin and

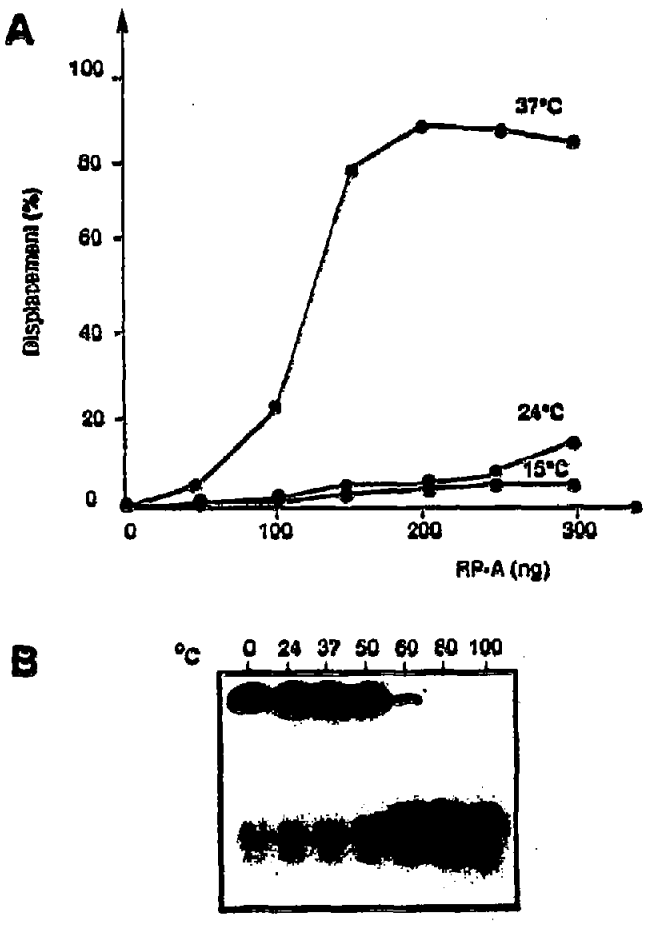


Fig. 3. Effect of temperature on the unwinding by calf thymus RP-A on the short (24mer) substrate. (A) DNA unwinding was performed at 15, 24 or 37°C as described in Materials and Methods and various amounts of calf thymus RP-A tested. (B) Stability of short substrate under experimental condition was tested in the absence of RP-A. Reaction mixtures were incubated for 30 min at various temperatures and analyzed as described for the unwinding assay.

chymostatin. *Buffer B:* 30 mM Tris-HCl (pH 8.0), 0.02% (v/v) Nonidet P-40, 0.125% (w/v) myo-inositol, 2 mM EDTA, 1 mM dithiothreitol and all the protease inhibitors mentioned in buffer A. *Buffer C:* 20 mM Tris-HCl (pH 8.0), 10% (v/v) glycerol, 0.02% (v/v) Nonidet P-40, 2 mM EDTA, 1 mM dithiothreitol and all the protease inhibitors mentioned in buffer A. *Buffer D:* as buffer C but without Nonidet P-40.

500 g of frozen calf thymus (from fetal and 6-month-old animals; the tissues were stored at −70°C until use) were suspended in 1500 ml of buffer A during 60 min and subsequently homogenized in a Waring blender for 5 min. The suspension was centrifuged at 10,000 × *g* for 30 min. After passage through several layers of cheesecloth, the cleared supernatant was loaded onto a 500 ml of phosphocellulose column equilibrated with buffer A. The flow-through was collected and immediately loaded onto a 180 ml DEAE-Sephacel column equilibrated with buffer B containing 100 mM KCl. The column was washed with 900 ml of buffer C containing 100 mM KCl. RP-A was eluted in one step with buffer B, containing 300 mM KCl and the fractions containing RP-A were brought to 800 mM NaCl. This solution was then loaded onto a 10 ml single-stranded DNA cellulose column equilibrated in buffer C containing 800 mM NaCl. The column was washed with 100 ml of buffer C containing 900 mM NaCl and RP-A eluted with 30 ml of buffer C containing 2.5 M NaCl. The fractions containing RP-A were pooled, extensively dialyzed against buffer C and loaded into a 1 ml FPLC Mono Q column equilibrated

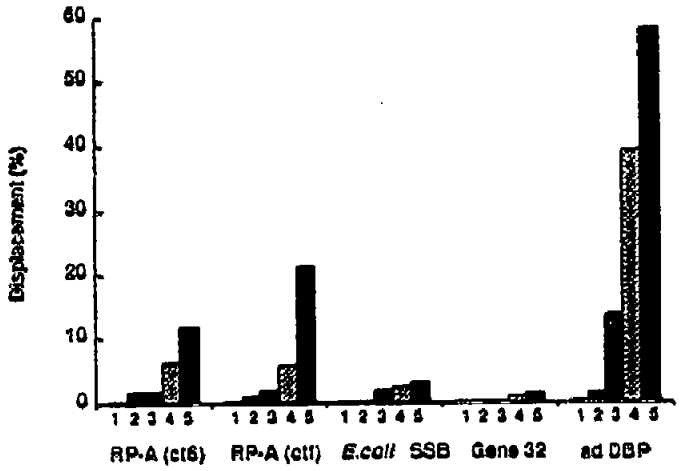


Fig. 4. DNA unwinding by calf thymus RP-A and by different single-stranded DNA binding proteins. DNA unwinding was performed with none (1), 50 ng (2), 100 ng (3), 200 ng (4) and 300 ng (5) of the following single-stranded DNA binding proteins: calf thymus RP-A from six-month-old animals (RP-A, ct6), fetal calf thymus (RP-A, ctf), *Escherichia coli* single-stranded binding protein (*E. coli* SSB), bacteriophage T4 gene 32 protein (gene 32) and adenovirus single-stranded DNA binding protein (ad DBP).

with buffer D. Elution of RP-A was performed with a linear gradient of 20 ml from 50 to 500 mM NaCl in buffer D. RP-A eluted at 250 mM NaCl and the active fraction were dialyzed against buffer D to remove NaCl and the protein stored in small aliquots in liquid nitrogen until further use.

2.4. *Preparation of DNA substrates*

A short substrate (24mer hybridized to M13mp8 DNA) and a long substrate (50 to 350mer hybridized to M13mp8 DNA) were as outlined for DNA helicases [12].

2.5. *Unwinding assay*

Unwinding of the short or the long substrate was carried out in a final volume of 25 μl containing 20 mM Tris-HCl (pH 7.5), 4% (w/v) sucrose, 8 mM dithiothreitol, 80 μg/ml bovine serum albumin, 10 ng DNA substrate (3000 cpm/pmol) and protein to be tested. Incubation was for 60 min at 37°C unless otherwise mentioned. The reaction was stopped and the product electrophoresed, autoradiographed and quantified as described [12].

## 3. RESULTS AND DISCUSSION

RP-A has so far been described in human cells [15–17], yeast *Saccharomyces cerevisiae* [2], *Xenopus laevis* eggs [13] and calf thymus [12] (Fig. 1). The 55 kDa polypeptide occurring in many RP-A preparations appears to be a degradation product of the 70 kDa subunit, since it reacts in an immunoblot with an antibody that was raised against the 70 kDa subunit (data not shown). As expected the calf thymus, RP-A has the same polypeptide composition as other eukaryotic counterparts, and their individual subunits are closely related since they crossreact immunologically (data not shown). The 32 kDa subunit is present as a doublet; one band thereof most likely represents the phosphorylated form of this subunit [8].

Titration of RP-A in a standard DNA helicase assay

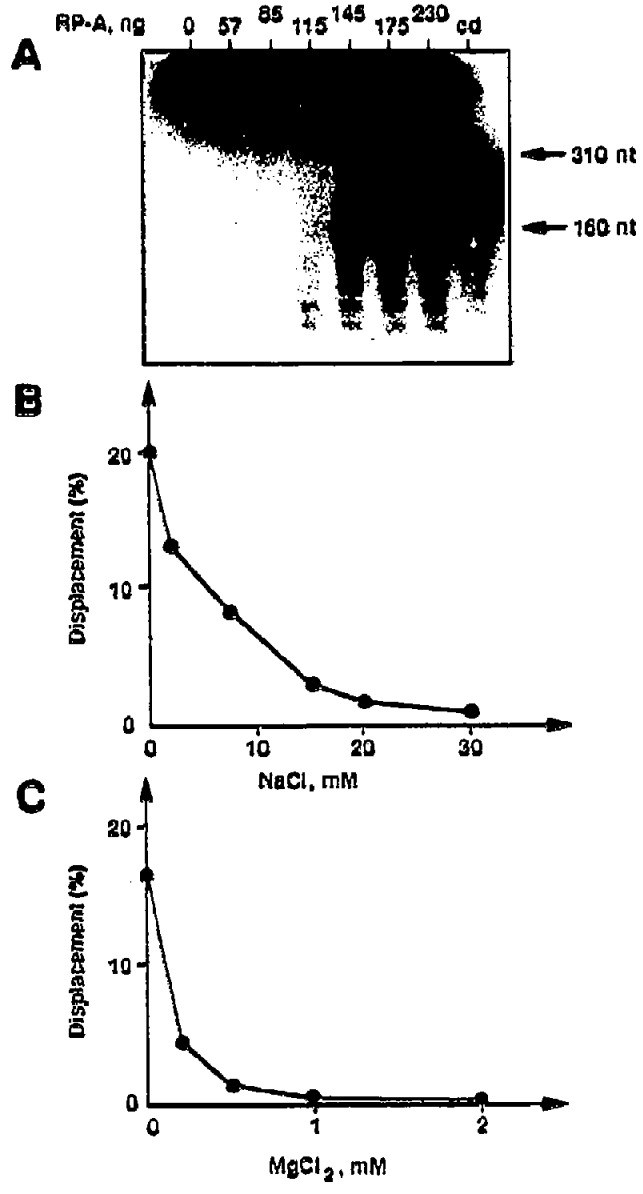


Fig. 5. DNA unwinding by calf thymus RP-A on the long (50–350mer) substrate. DNA unwinding was performed as described in Materials and Methods. (A) Titration of RP-A. cd = substrate alone boiled for 3 min. (B) Unwinding of the substrate by 145 ng RP-A in the presence of NaCl. The initial concentration of NaCl in the assay due to the substrate DNA and RP-A was 20 mM. (C) Unwinding of the substrate by 145 ng RP-A in the presence of $MgCl_2$.

indicated that a short (24mer) fragment can be displaced (Fig. 2A) at an amount of RP-A (100 ng, 0.88 nmol) that was sufficient to cover the single-stranded M13 DNA template (10 ng, 3.3 pmol of nucleotide), assuming that one molecule of RP-A covers 30 nucleotides [1]. Neither $MgCl_2$ nor ATP were required for the displacement indicating that the activity is DNA unwinding and not a *bona fide* DNA helicase activity (Fig. 2B). DNA unwinding was rapid since at saturating amounts of RP-A (300 ng) complete unwinding was achieved within 5 min (Fig. 2C).

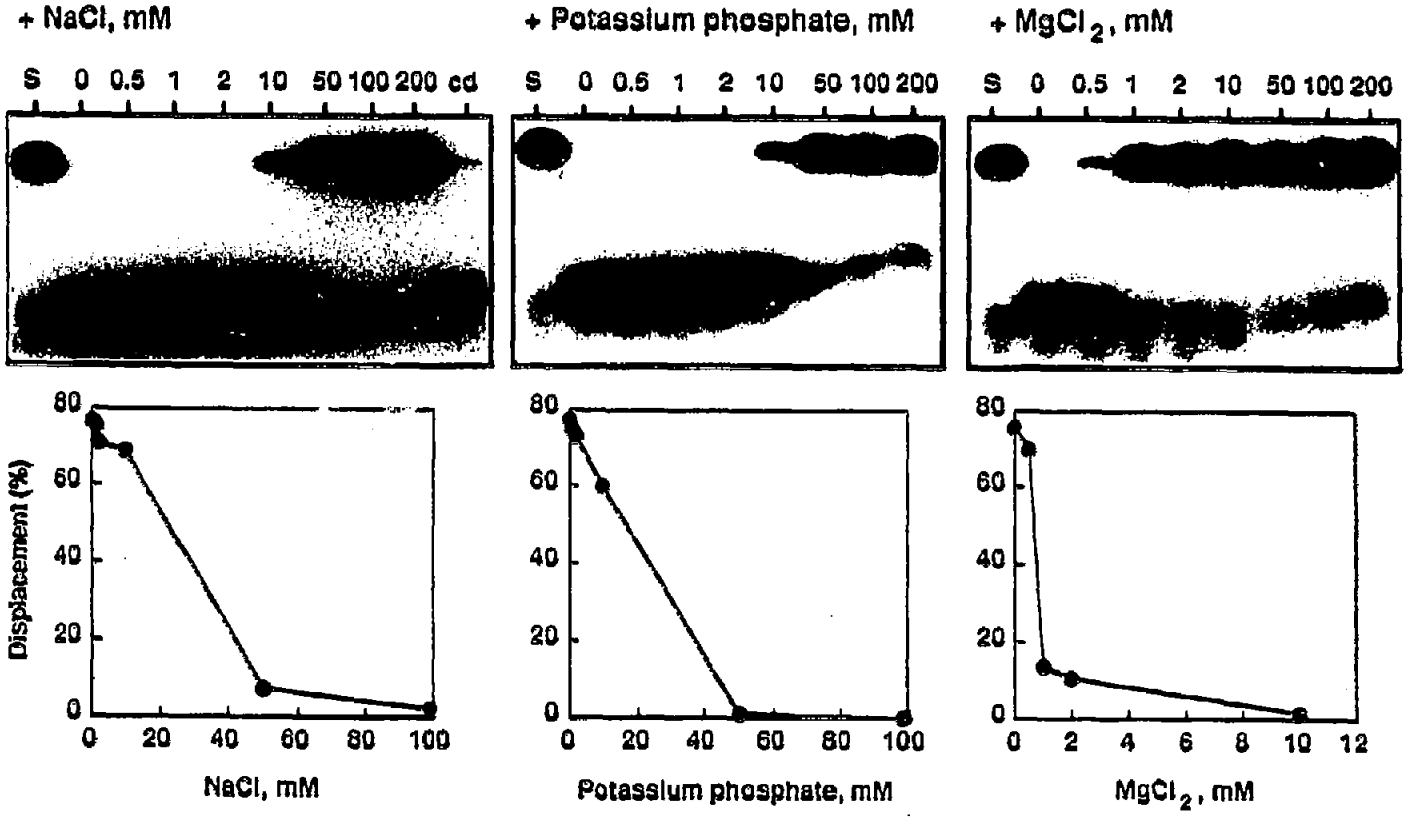


Fig. 6. Salt dependence of the DNA unwinding by calf thymus RP-A on the short (24mer) substrate. DNA unwinding was performed as described in Materials and Methods. The initial concentration of NaCl in the assay due to the substrate DNA and RP-A was 15 mM. Each assay contained 200 ng RP-A. S = substrate alone; cd = substrate alone boiled for 3 min.

Next, the effect of temperature was tested. DNA unwinding by RP-A could also be performed at 15 and 24°C. Unwinding at 15°C was 5% and at 24°C 10–15% to the extreme at 37°C (Fig. 3A). No unwinding was measured in the absence of RP-A at temperatures up to 60°C (Fig. 3B) indicating that the increase of temperature can facilitate unwinding but is not the reason for it.

DNA unwinding under these conditions could also be performed by RP-A from fetal calf thymus and by the adenovirus DNA binding protein, but not by single-stranded DNA binding proteins from *Escherichia coli* or from bacteriophage T4 (Fig. 4). Unwinding by the adenovirus DNA binding protein is not unexpected since it has been shown that strand displacement synthesis occurs in the presence of adenovirus DNA polymerase and DNA binding protein in the obvious absence of an added DNA helicase [18]. Fig. 5 shows that RP-A can also unwind longer substrates. For this a ladder of fragments from 50 to 350 bases was synthesized as described earlier [12] and used as a substrate for unwinding. RP-A had significant unwinding activity up to 350 basepairs (Fig. 5A) and this in the complete absence of $MgCl_2$ and ATP. The unwinding reaction on both short and long substrates was very sensitive to salt (Figs. 5 and 6). 50 mM NaCl, 50 mM potassium phosphate or 1 mM $MgCl_2$ inhibited the unwinding reaction more than 90% if tested with a short substrate (Fig. 6). The displacement of long substrates was even more sensitive to salt. 5 mM NaCl reduced unwinding to 50% and 10 mM to 25% of the unwinding in the absence of salt (Fig. 5B), while 0.2 mM $MgCl_2$, reduced unwinding to 26% and 1 mM to less than 2.5% compared the activity without $MgCl_2$ (Fig. 5C).

RP-A possesses an in vitro unwinding activity which might favour one function that has been proposed for this protein. It is a key protein that might interact at the origin of replication with origin binding proteins (e.g. with the origin recognition complex, ORP [19]), with DNA helicases, and with DNA polymerase α/primase. Its unwinding activity could provide a proper substrate for a cellular DNA helicase, that could subsequently act possibly in combination with RP-A. Indeed, a species specific stimulation of cellular DNA helicases by RP-A has been described in calf thymus [12]. Furthermore the phosphorylation status of the 32 kDa polypeptide is another possibility to selectively turn on and off the unwinding activity. Finally, interaction of RP-A during DNA synthesis with DNA polymerase α/primase or with DNA polymerase δ and ε holoenzymes (DNA polymerase, PCNA and RF-C) are other possibilities. These questions can now be addressed in the calf thy-

mus tissue with the three DNA polymerases $\alpha$, $\delta$ and $\epsilon$ [20], the four DNA helicases A, B, C and D [12] and the three auxiliary proteins RP-A, PCNA and RF-C [14].

*Acknowledgements:* This work was supported by the Swiss National Science Foundation (Grants 31.28592.90 and 31.30238.90), by the Swiss Cancer Society, by the Ciba-Geigy Jubiläumsstiftung, by the Bonizzi-Theler Stiftung, and by the Kanton of Zürich. We thank P.C. van der Vliet for a gift of adenovirus DNA binding protein.

## REFERENCES

[1] Kim, C., Snyder, R.O. and Wold, M.C. (1992) Mol. Cell. Biol. 12, 3050–3059.
[2] Brill, S.J. and Stillman, B. (1989) Nature 342, 92–95.
[3] Brill, S.J. and Stillman, B. (1991) Genes Dev. 5, 1589–1600.
[4] Heyer, W.-D., Rao, M.R.S., Erdile, L.F., Kelly, T.J. and Kolonder, R.D. (1990) EMBO J. 9, 2321–2329.
[5] Stillman, B. (1989) Annu. Rev. Cell. Biol. 5, 197–245.
[6] Kenny, M.K., Schlegel, U., Furneaux, H. and Hurwitz, J. (1990) J. Biol. Chem. 265, 9757–9761.
[7] Dornreiter, I., Erdile, L.F., Gilbert, I.U., von Winkler, D., Kelly, T.J. and Fanning, E. (1992) EMBO J. 11, 769–776.
[8] Din, S.-U., Brill, S.J. and Stillman, B. (1990) Genes Dev. 4, 968–977.
[9] Fotedar, R. and Roberts, J.M. (1992) EMBO J. 11, 2177–2187.
[10] Dutta, A. and Stillman, B. (1992) EMBO J. 11, 2189–2199.
[11] Tsurimoto, T. and Stillman, B. (1989) EMBO J. 8, 3883–3889.
[12] Thömmes, P., Ferrari, E., Jessberger, R. and Hübscher, U. (1992) J. Biol. Chem. 267, 6063–6073.
[13] Adachi, Y. and Laemmli, U.K. (1992) submitted.
[14] Podust, V., Georgaki, A., Strack, B. and Hübscher, U. (1992) submitted.
[15] Wobbe, C.R., Weissbach, L., Borowiec, J.A., Dean, F.B., Murakami, Y., Bullock, P. and Hurwitz, J. (1987) Proc. Natl. Acad. Sci. USA 84, 1834–1838.
[16] Wold, M.S. and Kelly, T.J. (1988) Proc. Natl. Acad. Sci. USA 85, 2523–2527.
[17] Fairman, M.P. and Stillman, B. (1988) EMBO J. 7, 1211–1218.
[18] Lindenbaum, J.O., Field, J. and Hurwitz, J. (1986) 261, 10218–10227.
[19] Bell, S.B. and Stillman, B. (1992) Nature 357, 128–134.
[20] Weiser, T., Gassmann, M., Thömmes, P., Ferrari, E., Hafkemeyer, P. and Hübscher, U. (1991) J. Biol. Chem. 266, 10420–10428.