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**Single stranded DNA binding proteins derive from hnRNP proteins by proteolysis in mammalian cells**

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**ABSTRACT**

As we have previously demonstrated, mammalian single stranded DNA binding proteins (ssDBP) and heterogeneous nuclear RNA binding proteins (hnRNP proteins) are antigenically and structurally related.

In this paper we show that ssDBP are specific proteolytic products of hnRNP core proteins. Proteolysis can be observed in crude extract, both total and nuclear and is not inhibited by the most commonly used protease inhibitors. Such phenomenon can be observed in HeLa cells, human fibroblasts and calf thymus extracts. A trypsin-like protease that cleaves purified hnRNP proteins to give ssDBP of  $M_r = 24-28$  Kd can be purified from HeLa cells. A precursor-product relationship can be established between hnRNP core proteins type A and an ssDBP of 24 Kd (UP1).

**INTRODUCTION**

Mammalian single stranded DNA binding proteins (ssDBP) and heterogeneous nuclear RNA binding proteins (hnRNP proteins) have long been considered as two independent protein groups. Both groups of proteins consist of a set of related polypeptides which are highly conserved among different species (1-7). While the first are thought to be involved in DNA replication as accessory proteins of DNA polymerase  $\alpha$  (1-3) the second, particularly the so called hnRNP core proteins (4,5) are believed to play a pivotal role in the post-transcriptional RNA processing cascade (6).

In a previous paper we have shown that single stranded DNA binding proteins (ssDBP) from both calf thymus and HeLa cells are antigenically and structurally related with some (if not all) of the corresponding hnRNP core proteins (7). One of the hypotheses raised by this observation was that ssDBP could derive from the larger hnRNP proteins by specific proteolysis. In this paper we present evidence that this is in fact the case. In effect ssDBP are

## Nucleic Acids Research

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not detectable as such in HeLa cell extracts, both nuclear and cytoplasmic, upon fractionation by SDS-PAGE and immunoblot analysis. The same is true for cultured human fibroblasts, while a certain amount of ssDBP is observed in calf thymus extracts. In all three cases, however, ssDBP are produced at the expenses of hnRNP proteins upon prolonged self-incubation of the extracts. The proteolytic cleavage occurs also in the presence of the most commonly used protease inhibitors. We have also isolated and partially purified from HeLa cells a specific protease which extensively converts hnRNP proteins into ssDBP. The protease, which is also detected in purified nuclei, has a trypsin-like mode of action.

### MATERIALS AND METHODS

#### Antibody preparation

Antibodies against a pure subset of calf thymus ssDBP were prepared in rabbit and purified as described in a previous paper (7). The titer of purified antibodies as determined by enzyme linked immunosorbent assay (ELISA) was about 1:100,000. Antibodies against a homogeneously pure ssDBP from HeLa cells ( $M_r$  24-26 Kd, see text) were produced in a 6-week-old Balb/c female mouse in the following way: the first intraperitoneal shot contained 40  $\mu$ g of antigen in 50  $\mu$ l of sterile saline solution plus 50  $\mu$ l complete Freund adjuvant. The second injection after five weeks, was like the first but with incomplete Freund adjuvant. Two more injections were done at one week intervals with the same amount of antigen but without adjuvant. The final antibody titer in the serum was higher than 1:12,000 as determined by the ELISA technique.

#### Buffers used

Buffer A: Tris HCl pH 8.8, 20mM; NaCl 50mM; EDTA 1mM; DTT 0.1mM; PMSF 1 mM;  $Na_2S_2O_5$  10 mM; glycerol 5%. Buffer T: K-phosphate pH 7.5, 20mM; DTT, 0.5mM; EDTA, 1mM; PMSF, 1mM;  $Na_2S_2O_5$  10mM; aminoacetonitrile 0.1mM; glycerol 20%. Buffer B: Hepes pH 7.5, 20 mM; KCl, 5 mM;  $MgCl_2$ , 0.5 mM; DTT, 0.5 mM; sucrose, 0.2 M. PBS: phosphate buffer saline.

#### Preparation of whole cell sonicates

Extracts were prepared by sonication as described in a previous paper (7) from fresh exponentially growing HeLa cells, human fibroblasts and calf thymus

tissue taken from a 4-month-old calf and immediately frozen in liquid nitrogen.

#### Preparation of HeLa cell nuclei

Procedure A: One liter of HeLa cells grown in suspension to a density of  $10^8$  cells/l were harvested, washed with PBS and with Buffer B and resuspended in 50 ml of the same buffer without sucrose. After standing 10 min in ice, cells were broken in a Dounce homogenizer. Nuclear and cytoplasmic fractions were separated by low speed centrifugation (800xg) at 4°C. Procedure B: For some experiments (see text) nuclei, prepared as in procedure A, were further purified by following the procedure of Zieve and Penman (8). Purified nuclei were broken by sonication as previously described.

#### Incubation of sonicates

Unless otherwise stated in the text, whole cell sonicates or sonicated nuclei (0.3 - 0.5 mg of protein per sample) were incubated 15 hrs at 30°C in Buffer A or in Buffer T as specified in the text. The incubation was stopped by the addition of Laemmli sample buffer (10), the samples were heated for 2 min at 90°C and immediately fractionated by SDS-PAGE (10) for immunoblot analysis.

#### Preparation of hnRNP core proteins

hnRNP core proteins ( $M_r = 32-38$  Kd) were prepared from purified HeLa cell nuclei using the standard pH8-diffusion technique followed by sucrose gradient centrifugation to obtain the 40S hnRNP particles (4,9).

#### Chromatographic purification of hnRNP proteins

We have developed a straightforward procedure for the preparative isolation of hnRNP proteins from large amounts of HeLa cells. The details of the procedure will be published elsewhere (Eur. J. Biochem., submitted). In essence it entails homogenization of cells by sonication and removal of nucleic acids by filtration on DEAE-cellulose in 0.35M K-phosphate (pH7.5) buffer. The nucleic acid-free protein solution is applied onto an ssDNA cellulose which is first washed with a 20 mM to 1M K-phosphate gradient (which removes most of the bound proteins but does not remove hnRNP proteins) and then with a 50 mM to 1M NaCl gradient. hnRNP proteins elute between 0.5 and 0.8M NaCl. Further purification and fractionation is obtained by chromato-

graphy on hydroxyapatite where hnRNP proteins elute between 0.15 and 0.25 M K-phosphate.

### Partial purification of the endogenous HeLa protease

A protease that cleaves hnRNP proteins to discrete smaller polypeptides was detected during the chromatographic purification of hnRNP proteins (see previous section). The purification procedure and the initial characterization of this activity will be described elsewhere (Eur. J. Biochem., submitted). Briefly the first steps of the purification procedure are the same as those described in the previous section for the hnRNP proteins; the protease is eluted from the ssDNA cellulose column by the NaCl gradient between 0.3 and 0.6 M NaCl (partially overlapping the peak of hnRNP proteins). The protease is further purified on hydroxyapatite where it elutes between 0.15 and 0.2 M K-phosphate (about the same molarity that elutes the bulk of hnRNP proteins).

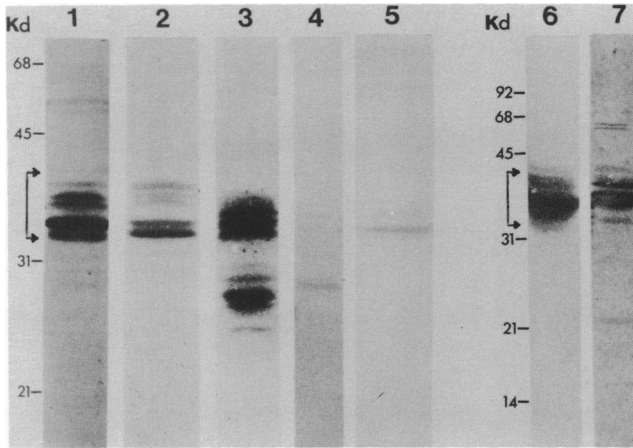
### Separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

Electrophoretic fractionation in 12% polyacrylamide of total sonicates and of purified proteins, electrophoretic transfer to nitrocellulose membranes and detection with antibodies were performed as previously described (7). All experiments were performed in antibody excess conditions.

## RESULTS

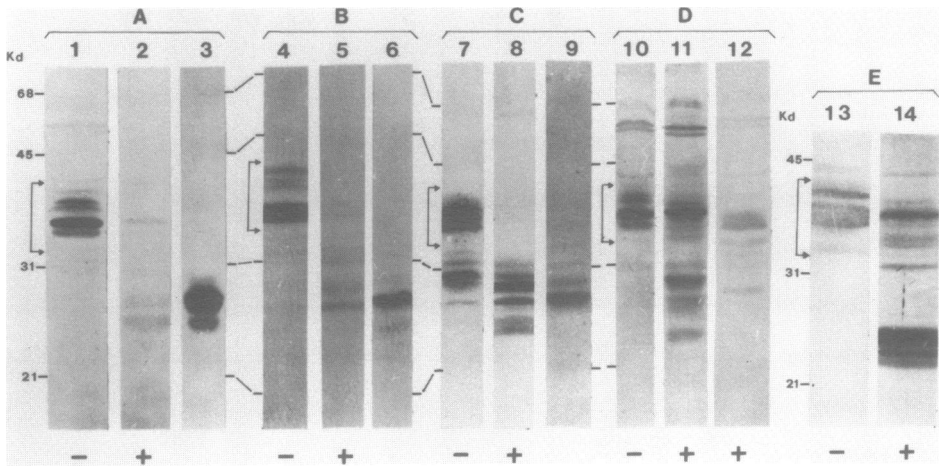
### Immunologic detection of ssDBP and hnRNP proteins in crude extracts

In a previous paper we reported that antibodies prepared against a pure subset of single stranded DNA binding proteins (ssDBP) from calf thymus recognize, in crude extracts of calf thymus and HeLa cells, not only proteins corresponding to the antigen but also and to a greater extent a family of higher molecular weight polypeptides. Such proteins were identified as the hnRNP core proteins by several criteria (7). These first experiments also indicated that, contrary to what is observed in calf thymus extracts, HeLa cells and human fibroblast extracts show very little, if any, protein reacting with antibodies in a molecular weight range corresponding to that of the described mammalian ssDBP (24-28 Kd). In order to confirm and strengthen these observations, the experiments were repeated using, besides the



**Fig. 1** - Immunologic detection of hnRNP proteins in total cell sonicates with antibodies against calf thymus and HeLa cell ssDBP, raised in rabbit and in mouse respectively. Extract preparation, SDS-PAGE and immunoblot analysis were as described in Materials and Methods. HeLa cells and calf thymus sonicates (about 0.3 mg/lane) stained with antibodies made in rabbit (lanes 1 and 3) and with antibodies made in mouse (lanes 2 and 4). Lane 5: human fibroblast sonicate (about 100  $\mu$ g) stained with rabbit antibodies. 40S hnRNP core proteins (about 10  $\mu$ g/lane) stained with mouse antibodies (lane 6) and with rabbit antibodies (lane 7). Brackets indicate the expected position of hnRNP core proteins.

previously described rabbit antibodies against calf thymus ssDBP, antibodies made in mouse (see Materials and Methods) against a pure single stranded DNA binding protein from HeLa cells ( $M_r = 24-26$  Kd) purified by the standard techniques (1,3). This protein is strongly homologous to calf thymus UP1 (1) on the basis of peptide mapping and partial aminoacid sequence (paper in preparation). The results of these experiments are shown in Fig. 1. As it can be seen (Fig. 1, lanes 1 and 2) mouse antibodies, like rabbit antibodies (both used in excess), react primarily with hnRNP core proteins ( $M_r = 32-38$  Kd) in western blots of HeLa cell sonicates. In addition it is evident that only calf thymus extracts contain appreciable amounts of reacting proteins in the 24-28 Kd range (ssDBP) (Fig. 1, lanes 3 and 4). As expected, both antibodies strongly stain 40S hnRNP core proteins purified by the "canonical" procedures (see Materials and Methods) (Fig. 1, lanes 6-7). The specificity of mouse antibody is, however, slightly different from that of the rabbit antibodies



**Fig. 2** - Endogenous proteolytic digestion of hnRNP proteins following incubation of total cell or nuclear sonicates. Extract preparation, digestion conditions and immunoblot analysis were as described in Materials and Methods. All membranes, except those in **Panel B** were treated with antibodies raised in rabbit (see text). **Panels A** and **B**: Total HeLa cell sonicates (about 0.5 mg of protein/lane) stained with antibodies raised in a rabbit and mouse respectively. **Panel C**: Total calf thymus sonicates (0.5 mg of protein/lane). **Panel D**: HeLa cell nuclear sonicates (0.5 mg of protein/lane). (-): unincubated; (+): incubated. **Panel E**: 10  $\mu$ g of 40S hnRNP core proteins, purified by the standard procedures (4,9), (-): undigested; (+): partially digested with trypsin as previously described (3). **Lanes 3** and **6**: 5  $\mu$ g of ssDBP purified from HeLa cells (see text) stained with rabbit and mouse antibodies respectively. **Lane 9**: 5  $\mu$ g of ssDBP from calf thymus (3). **Lanes 10-11**: Extracts of nuclei prepared following **Procedure A** (see Materials and Methods). **Lane 12**: Extract of nuclei prepared following **Procedure B** (see Materials and Methods). Brackets indicate the expected position of hnRNP core proteins.

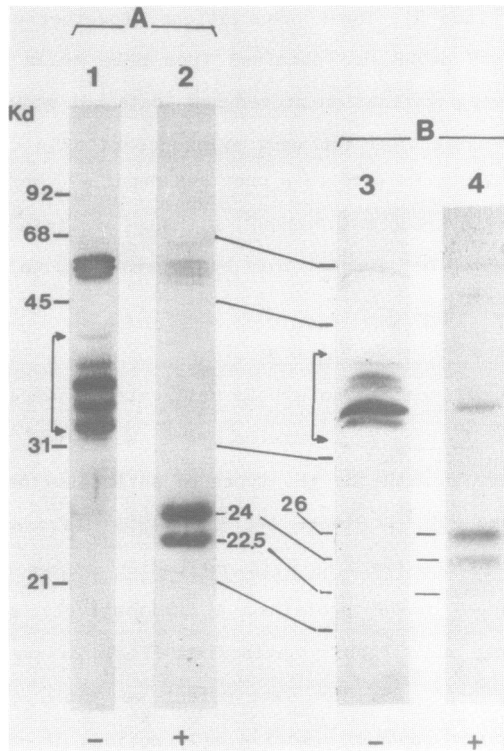
previously described, as indicated by the different relative intensity of hnRNP core protein bands ( $M_r = 32-38$  Kd) and by the fact that mouse antibodies (against the 24-26 Kd HeLa ssDBP) do not recognize in HeLa sonicates a set of two-three bands at 53-55 Kd (see ahead) which is on the contrary recognized by the antibodies against calf thymus ssDBP made in rabbit (see Fig. 1, lanes 1-2, 6-7). Also mouse antibodies against HeLa ssDBP seem to have a lower affinity for calf thymus proteins (Fig. 1, lanes 3-4); this point however is presently being further investigated. Finally it can be seen that human fibroblast extracts give a pattern which is qualitatively similar to that of

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HeLa cells (Fig. 1, lane 5). These observations, together with the fact that very small amounts of ssDBP are obtained from HeLa cells compared to calf thymus by the standard purification procedures (data not shown), raise several questions about the origin and the very existence of this class of proteins. One of the hypotheses put forward in a previous paper (7) was that ssDBP could be in vitro proteolytic products of hnRNP. Although preliminary experiments on calf thymus extracts did not support this hypothesis (7), we decided to submit it to a more thorough test.

#### Endogenous proteolysis in crude extracts

In the first experiments, whole HeLa cell extracts were incubated for 15 hrs at 30° C in Buffer A, proteins were separated by SDS-PAGE and immunologically detected with the two types of antibodies described above. As it can be seen, (see Fig. 2, lanes 1-2 and 4-5) this treatment causes the generation of polypeptides reacting with antibodies in the Mr range 24 to 28 Kd at the expenses of hnRNP proteins. A qualitatively similar result is obtained by staining with rabbit antibodies (Fig. 2, panel A) and mouse antibodies (Fig. 2, panel B). The same effect was observed in calf thymus extracts, which already contain reactive polypeptides in the Mr range 24-26 Kd. Incubation of these extracts for 15 hrs at 30°C in Buffer A causes in fact the complete disappearance of hnRNP bands (32-38 Kd) and the corresponding increase of low Mr bands (Fig. 2, panel C, lanes 7-8). No significant difference in the degradation pattern was observed when the extracts were incubated in Buffer A or in Buffer T, both containing three most commonly used protease inhibitors, and in Buffer B without protease inhibitors (not shown). The identity of the low Mr polypeptides, generated during incubation, with the mammalian ssDBP is indicated by the same mobility in SDS-PAGE (Fig. 2, lanes 2-3, 5-6 and 8-9) and by the reaction with specific antibodies. Some immunoreactive bands generated during incubation of calf thymus extracts line up in SDS-PAGE with known and characterized ssDBP species at 26 Kd and 28 Kd we described in a previous paper (3) (Fig. 2, panel C, lanes 8-9). It should be observed that also the low Mr bands, already present in calf thymus extracts, (see Fig. 2, Panel C, lane 7), which correspond to the highest Mr ssDBP species we described in a previous paper (3), are further digested with



**Fig. 3** - Specific proteolysis of purified hnRNP proteins by a HeLa cell endogenous protease. **Panel A:** hnRNP proteins purified chromatographically as described in the text. **Panel B:** 40S hnRNP core proteins purified according to Beyer et al. (4). 5  $\mu$ g of proteins were incubated with limiting amounts of the partially purified HeLa cell endogenous protease for 15 hrs at 30°C in Buffer T. (-): unincubated; (+): incubated. SDS-PAGE and immunoblot analysis were performed as described in Materials and Methods. Brackets indicate the expected position of hnRNP core proteins.

the production of a 24 Kd band most likely corresponding to Herrick and Alberts' UPl protein (1). On the basis of these data it can be concluded that crude cellular extracts contain a protease which specifically degrades hnRNP proteins to smaller polypeptides, some of which correspond to ssDBP.

Intracellular localization of protease

Since hnRNP proteins are described as nuclear proteins (4,8,11,12) and ssDBP are supposed to work inside the nucleus, we asked the question whether proteolytic degradation of hnRNP could be observed also in nuclear extracts.



HeLa cell nuclei and nuclear extracts were prepared as described (see Materials and Methods, procedure A). After incubation for 24 hrs at 30° C in Buffer A the proteins were separated by SDS-PAGE and immunologically detected with rabbit antibodies. As it can be seen (Fig. 2, panel D, lanes 10-11), extensive degradation is observed also in nuclear extracts with 28 to 22 Kd discrete bands being generated. The 26 Kd and 24 Kd bands line up perfectly with the pure HeLa cell ssDBP fraction prepared by the standard purification procedures (1,3) (Fig. 2, lanes 3 and 6). In order to rule out the possibility that the digestions observed in nuclear extracts could be due to cytoplasmic contaminations the assay was repeated on nuclei purified in a more rigorous way (see Materials and Methods, procedure B). Also in this case (see Fig. 2, panel D, lane 12) significant degradation is observed. Thus it can be concluded that the localization of the protease that converts hnRNP proteins into ssDBP is most likely nuclear. We will address this point again in the Discussion.

The proteolysis observed in crude and in nuclear extracts is probably due to a trypsin-like enzymatic activity, since the degradation pattern resembles that produced by limited digestion with trypsin of purified 40S hnRNP core proteins (see Fig. 2, panel E, lanes 13-14).

#### Partial purification of protease

A protease that cleaves purified hnRNP proteins in vitro in a way similar to that observed in total or nuclear extracts was detected in the course of the chromatographic purification of hnRNP proteins outlined in Materials and Methods. The details of the preparative purification of hnRNP proteins will be reported elsewhere (Eur. J. Biochem., submitted). Here it is worth mentioning that this method yields large amounts (about 0.3 mg/g of cells) of a sextet of proteins ( $M_r = 32-38$  Kd) corresponding to the described hnRNP core proteins (4,9) plus appreciable amounts of two-three closely spaced protein bands with  $M_r = 53-55$  Kd (see Fig. 3, lane 1). We have several evidences indicating that the latter, so far unreported, proteins are structurally related to at least some of the hnRNP core proteins (manuscript in preparation). A puzzling observation made in the course of the purification was that a protease that cleaves hnRNP proteins to smaller products corresponding to ssDBP appears to

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follow the bulk of hnRNP proteins both on ssDNA cellulose and on hydroxyapatite (see Materials and Methods). Proteolysis of partially purified hnRNP proteins was similar to that observed in crude extracts since it produced a ladder of discrete polypeptides with  $\bar{M}_r = 22-28$  Kd. By using this type of degradation as a specific assay we were able to considerably purify the endogenous protease which is presently being characterized in more detail in our laboratory. When hnRNP proteins purified by chromatography as described above or by the standard procedures (4,9) are incubated at 30°C for 15 hrs with limiting amounts of the partially purified protease the result shown in Fig. 3 is obtained. hnRNP core proteins ( $\bar{M}_r = 32-38$  Kd) are quantitatively converted into a ladder of smaller polypeptides with  $\bar{M}_r = 26$  Kd, 24 Kd and 22.5 Kd. As it can be seen, in this particular experiment the digestion conditions (which are not easy to calibrate) lead to the accumulation of 24 and 22.5 Kd bands in the case of chromatographically purified hnRNP proteins (Fig. 3, panel A) and of 26 and 24 Kd bands with only traces of the 22.5 Kd band in the case of "canonical" 40S hnRNP core proteins (Fig. 3, panel B). The 26 Kd and 24 Kd proteins most likely correspond to the HeLa cell ssDBP described in the previous sections.

### DISCUSSION

On the basis of the data presented in this paper we conclude that mammalian cells contain a trypsin-like protease that cleaves hnRNP proteins to smaller products some of which correspond to the single stranded DNA binding proteins ( $\bar{M}_r = 24-28$  Kd) described by us and by other authors (1-3). Our data indicate that the protease is most likely located in the nucleus and this finding is in accordance with the fact that both the substrate (hnRNP) and the presumptive products (ssDBP) of this enzyme are reported as being mostly nuclear proteins (2,4,9). However, similarly to what often happens with many bona fide nuclear proteins, also in this case some leakage in the cytoplasm was observed during the preparation of nuclei (data not shown). With regard to the details of the mechanism of hnRNP  $\longrightarrow$  ssDBP conversion only a few hypotheses can presently be put forward. For example the different ssDBP, which have been shown by us and by other authors to be a family of

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antigenically and structurally related proteins (2,3), could be generated by a single cleavage event on different hnRNP core proteins which, on the other hand, are themselves a family of related polypeptides (4-5,9,13). Alternatively they might constitute the ladder products of a proteolytic cascade affecting to a similar extent all hnRNP core proteins. Our data seem to indicate that at least in crude extracts both phenomena take place. In addition some of the polypeptides in the 26-28 Kd range seem to be products of proteolysis of the 53-55 Kd proteins that copurify with hnRNP core proteins (paper in preparation). However, at least in the case of the calf thymus 24 Kd ssDBP (UP1) (1), whose 195 aminoacid sequence has recently been published (14), it is now possible to establish a clearcut precursor-product correlation with some of the hnRNP core proteins. In fact it has been found that the aminoacid sequence of certain peptides from HeLa cell hnRNP core proteins type A1/A2 ( $M_r = 32-34$  Kd) perfectly overlaps the COOH-end of UP1 (14) and extends further with a sequence very rich in glycine ( $\approx 50\%$ ) (K. Schäfer and K. Beyreuther, University of Bochum; K. Williams, Yale University, personal communications). It appears therefore that the 24 Kd ssDBP (UP1) can derive from the homologous hnRNP core proteins A1/A2 (which are extremely conserved proteins) by a specific proteolytic cut at aminoacid 195 which removes 8-10 Kd at the COOH-end of the protein. This conclusion is confirmed by some preliminary gene cloning and sequencing studies currently under way in our laboratory which demonstrate that the nucleotide sequence of UP1 (24 Kd) is part of a longer open reading frame coding for a protein of about 32 Kd with an aminoacid composition identical to that of hnRNP core protein A1 (9) (paper in preparation). It is interesting to note that the cleavage that produces UP1 occurs at an arginine (aa 195) in close proximity (14) of a second modified arginine (aa 193: dimethylarginine) which could therefore constitute a recognition signal for the trypsin-like protease described in this paper. Furthermore by comparing the aminoacid composition of UP1 (1,2,14) and of hnRNP core proteins A1/A2 (4,9) it becomes evident that cleavage at aminoacid 195 removes a region of the molecule which, being extremely rich in glycine ( $\approx 50\%$ ), must constitute a different structural domain with respect to the UP1 portion of the polypeptide. These two observations rise the fascinating

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## Nucleic Acids Research

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possibility that the hnRNP A1/A2 to UP1 cleavage might play a physiological role in the control of the interaction of these proteins with RNA during processing. In addition an autonomous physiological role of ssDBP is suggested by the fact that these proteins are endowed with a unique functional property, stimulation of DNA polymerase  $\alpha$  (1,3) which is lacking in hnRNP core proteins (paper in preparation). If this were the case a situation similar to that described for some polyprotein systems (15) could be envisaged. Very intriguing in this respect is the co-purification of the protease with hnRNP protein (see text) and our recent observation that some protease is found also associated with the 40S hnRNP particles purified according to Beyer *et al.* (4) (data not shown). Both observations seem to indicate a close physical interaction between protease and hnRNP proteins with a possible functional significance.

It is clear however that a more detailed description of the hnRNP  $\rightarrow$  ssDBP conversion demands a better characterization of the two groups of proteins in terms of sequence, homology and structure.

In spite of all the fascinating possibilities discussed above, the biological significance of hnRNP  $\rightarrow$  ssDBP conversion is far from established. Our observations in fact raise the possibility that ssDBP might not be "real" but might be generated only in vitro upon breakage of the cell and during protein purification. This contention however seems to be opposed by the fact that fresh calf thymus sonicates contain significant amounts of ssDBP and that some ssDBP can be purified from HeLa cells even under conditions that should eliminate proteolysis. On the other hand the abundance of ssDBP in calf thymus (compared to HeLa) is rather disturbing since it could simply reflect a high level of endogenous proteolysis in resting tissues. In this connection however it is worth mentioning that no difference was observed in the relative ratio between ssDBP and hnRNP when exponentially growing and stationary cells (HeLa or human fibroblasts) were compared (data not shown). In any case it is possible that the massive degradation of hnRNP we observe under certain in vitro conditions is an amplification of a physiological phenomenon that occurs also in vivo but in a strictly controlled fashion.

In order to establish whether ssDBP are real and have a role in DNA

replication we are undertaking a series of experiments aimed at checking a possible inhibitory effect of our antibodies on DNA replication in permeabilized cells or in nuclear matrix preparations.

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#### ABBREVIATIONS

PMSF: phenylmethylsulfonyl fluoride. ssDBP: single stranded DNA binding proteins. hnRNP: heterogeneous nuclear RNA binding proteins. DTT: dithiothreitol. SDS-PAGE: sodium dodecylsulfate polyacrylamide gel electrophoresis.

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