Mammalian single-stranded DNA binding proteins and heterogeneous nuclear RNA proteins have common antigenic determinants

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ABSTRACT

Antibodies were raised in rabbit against a pure subset of calf thymus single-stranded DNA binding proteins (ssDBPs) and purified by affinity chromatography on antigen-Sepharose. In Western blot experiments these antibodies were shown to react to the same extent with the whole family of bovine ssDBPs, as well as with ssDBPs from HeLa cells. When used to stain total cell extracts from both calf thymus and HeLa cells the antibodies reacted only with bands corresponding to the ssDBPs and with a set of bands of higher molecular weight, whose electrophoretic pattern matched that of the 40S hnRNP core proteins. In effect we observed that purified 40S hnRNP core proteins from HeLa cells were strongly reactive with the antibodies. Moreover after partial tryptic digestion HeLa cells ssDBPs and hnRNPs produced immunoreactive fragments of the same molecular weight and isoelectric point. Extensive structural homologies can thus be evidenced between these two classes of proteins, which share the property of selective binding to single-stranded nucleic acids.

INTRODUCTION

Single-stranded DNA binding proteins (ssDBPs) or helix de-stabilizing proteins have been isolated from several mammalian tissues (1-5). In calf thymus, mouse myeloma and HeLa cells the ssDBPs appear to be a family of related molecular species with apparent M_r ranging between 24,000 and 30,000. In all cases partial digestion with trypsin reveals an underlying extensive structural homology among the different forms. The nature and the origin of this physical heterogeneity is still unclear although many explanations have been proposed (1,4).

In previous publications we showed that calf thymus ssDBPs are heterogeneous also for what concerns functional properties such as stimulation of DNA polymerase α and lowering of poly (d(AT)) melting temperature (3,5). Preliminary studies carried

out in our laboratory with antibodies made in mouse against a
subset of calf thymus ssDBPs demonstrated that all the different
polypeptides shared antigenic determinants and that a cross-
reacting, higher molecular weight species (31,000-32,000) could
be evidenced in partially purified preparations (5).

We report here our most recent studies with antibodies made
in rabbit against calf thymus ssDBPs and purified by affinity
chromatography on antigen-Sepharose. These experiments con-
firmed that in both calf thymus and HeLa cells all ssDBPs poly-
peptides share antigenic determinants, in fact calf thymus and
HeLa cells ssDBPs are strongly cross-reactive. In addition we
observed that in crude extracts of both calf thymus and HeLa
cells, a set of polypeptides in the $\underline{Mr}$ range 30,000-40,000
strongly and specifically react with the purified antibodies.
In this paper we show that the cross-reactive polypeptides in
the 30,000-40,000 molecular weight range can be identified with
the core proteins of the 40S heterogeneous nuclear RNA parti-
cles (hnRNP), a family of molecules believed to be involved in
the stabilization, transport and post-transcriptional process-
ing of unique sequence transcripts, described independently and
considered so far unrelated to the former (6-9). The biological
significance of these observations will be discussed.

EXPERIMENTAL PROCEDURES
Antigen preparation
   The antigen used for immunization was a pure subset of calf
thymus ssDBPs (pool 0.5 MIC) prepared as described in a pre-
vious paper (5) and corresponding by several criteria to pro-
tein UP1 (M) of Herrick and Alberts (1).
Rabbit immunization
   A New Zealand White rabbit received six intramuscular injec-
tions at two weeks' intervals. Each shot contained 200 µg of
antigen in 0.5 ml of sterile saline solution plus 0.5 ml of
complete Freund adjuvant. The final titre was about 1:100,000
as determined by the enzyme-linked immunosorbent assay (ELISA)
technique.
Antibody purification
   Twenty ml of immune serum were purified by ammonium sulphate

precipitation and affinity chromatography on antigen-Sepharose
as described by Shapiro et al. (10) and resuspended in the same
initial volume of newborn calf serum.

Crude extract preparation

The total cell sonicates were prepared as follows: 1 g (wet
weight) of HeLa cells or calf thymus was suspended in 10 ml of
Tris-HCl, pH 8.0, 10 mM; NaCl 50 mM; DTT 1 mM; glycerol 5%, con-
taining phenylmethylsulfonylfluoride (PMSF), 1 mM and sodium
bisulfite, 10 mM. The suspension was sonicated in ice three
times for 15 seconds at 100 W in ice and then centrifuged 10 min
at 20,000 x g. Aliquots of the supernatant (about 1 mg of pro-
teins) were concentrated by adding 5 volumes of cold acetone,
after 30 min in ice the solution was centrifuged 10 min at
12,000 rpm and the protein pellet was resuspended directly with
SDS-polyacrylamide gel electrophoresis sample buffer.

Preparation of ssDBPs from HeLa cells

ssDBPs were prepared from 30 g (wet weight) of frozen HeLa
cells by exactly the same procedure as that described for calf
thymus ssDBPs (5). The protein so obtained closely resembles
calf thymus proteins from all points of view (manuscript in pre-
paration).

Preparation of 40S hnRNP particles

hnRNP particles were purified from 3 g of exponentially grow-
ing HeLa cells according to Beyer et al. (6). Sucrose gradient
fractions containing 40S particles were pooled and the aliquots
to be applied to the gel were concentrated with acetone as de-
scribed above.

Gel electrophoresis and Western blot analysis

12% SDS-polyacrylamide gel electrophoresis was performed ac-
cording to Laemmli (11) on 1.5 mm thick slabs. Proteins were
then transferred to a nitrocellulose sheet, stained with tolui-
dine blue and immunologically detected as described by Towbin
et al. (12). A 1:1000 dilution of purified antibodies and a
1:1000 dilution of peroxidase-conjugated goat anti-rabbit IgG
(Miles-Yeda) were used.

Partial digestion with trypsin and analysis of fragments by two-dimensional electrophoresis

2-5 µg of proteins were digested with 0.03 IU of trypsin (Sig-

ma, 230 IU/mg) in Tris-HCl, pH 8.8, 20 mM; NaCl 10 mM; EDTA
0.1 mM; glycerol 10% in 30 μl final volume. Incubation was at
16°C for 30 min, the reaction was stopped by addition of the ap-
propriate amount of soybean trypsin inhibitor and the fragments
were separated by two-dimensional electrophoresis in the follow-
ing way. The first dimension (isoelectric focusing) was perform-
ed horizontally on 1 mm thick 6% polyacrylamide slab gels con-
taining 2% Ampholine pH 3.5-10 (13). 5 μg of each sample were
applied on the prefocused gel and run for 150 min at 10 W con-
stant power on an LKB Ultrophor apparatus cooled at 8°C. After
isoelectric focusing the gel was fixed in 20% TCA for 30 min,
rapidly stained with 0.3% Coomassie Brilliant Blue G-250 in 45%
methanol, 10% acetic acid and 0.5% $CuSO_4$ and destained with 25%
methanol, 10% acetic acid and 0.5% $CuSO_4$. The second dimension
was performed on 12% SDS-polyacrylamide vertical slab gels with
3% polyacrylamide stacking gel according to Laemmli (11). The
first dimension strip was cut out from the focusing gel, washed
10 min with distilled water, equilibrated 30 min with SDS sample
buffer and applied in direct contact with the dry stacking gel
surface. SDS-polyacrylamide gel electrophoresis was run over-
night on an LKB 2001 vertical electrophoresis unit with a power
supply setting of 70 V and 10 mA. Proteins were then blotted on-
to nitrocellulose and treated as described above.

RESULTS

High titre antibodies against purified calf thymus ssDBPs were
obtained in rabbit and subsequently purified by affinity chroma-
tography on antigen-Sepharose, as described in Experimental Pro-
cedures. As previously observed with antibodies raised in mouse
(5), these antibodies recognized all the different polypeptides
which constitute this family of proteins (see Fig. 1, lane 1).
We also observed that they had the same affinity for both the
antigen and the ssDBPs from HeLa cells (data not shown). This re-
sult, together with their striking physicochemical similarity
(manuscript in preparation) indicates a high degree of conser-
vation of these proteins in mammals.

In an attempt to better characterize these proteins and to
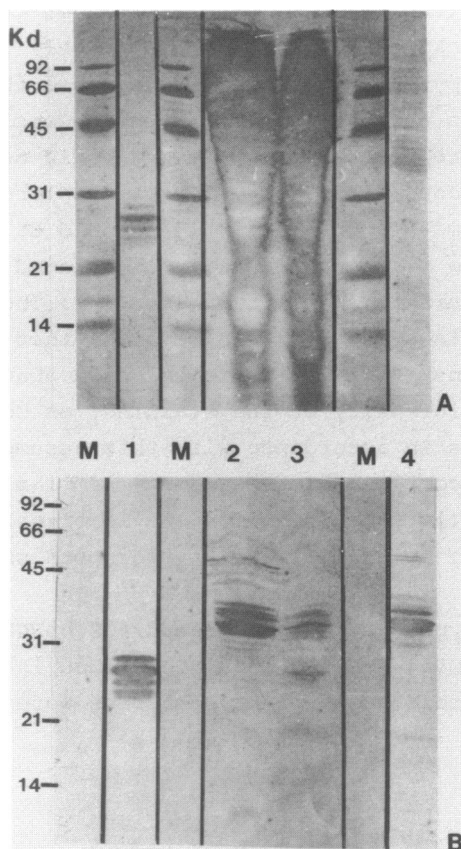understand their relationship with the high molecular weight

Fig. 1 - Immune cross-reactivity between calf thymus ssDBPs and 40S hnRNP core proteins. For the experimental details, see Experimental Procedures.
Panel (A): nitrocellulose sheet stained with toluidine blue.
Panel (B): the same sheet stained with antibodies.
Lane (1): calf thymus ssDBPs (antigen) 10 µg; lane (2): HeLa cell sonicate, 1 mg; lane (3): calf thymus sonicate, 1 mg; lane (4): 40S hnRNP proteins, 10 µg; (M): molecular weight markers; Kd: Kilodalton.

cross-reactive species previously observed (5), purified antibodies were used to stain total cell sonicates (of both calf thymus and HeLa cells) fractionated on SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose. The results, shown in Fig. 1, lanes 2 and 3, reveal that the antibodies react mostly with a set of bands with apparent M_r between 30,000 and 40,000 and also, though to a lower extent, with polypeptides of

Mr 20,000-30,000 (the same Mr range as that of the antigen
(lane 1)). The lack of reaction with other cell proteins, al-
beit present in appreciable amounts since more than 1 mg of pro-
tein was loaded on the wells, indicates a specificity of these
antibodies for a restricted group of antigenic determinants.

Three sets of considerations led us to explore the possibil-
ity that the 30,000-40,000 polypeptides could be identified with
the 40S hnRNP core proteins, a family of related polypeptides
with underlying structural similarities (14). Their electro-
phoretic pattern is strongly reminiscent of that of the 40S
hnRNP core proteins (6-9,14). Their relative abundance, as es-
timated from the intensity of the staining, as compared to that
of the antigen, is in accordance with this assumption (7). Also,
ssDBPs and hnRNP core proteins bear many similarities in amino
acid composition (1,4,6,7,9) and in one important physicochemi-
cal property, i.e. selective affinity for both ss-DNA and RNA
(1,8).

To test this hypothesis, we prepared 40S hnRNP complexes from
exponentially growing HeLa cells as described in Experimental
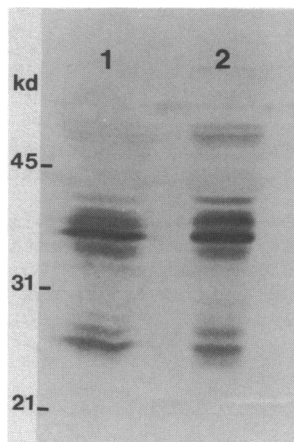Procedures. The proteins of hnRNP particles were separated on

Fig. 2 - Incubation of calf thymus sonicate at 30°C in the ab-
sence of protease inhibitors. The experiment was performed as
in Fig. 1 (Panel B).
Lane (1): calf thymus sonicate prepared as in Fig. 1, 1 mg;
lane (2): calf thymus sonicate prepared in the absence of pro-
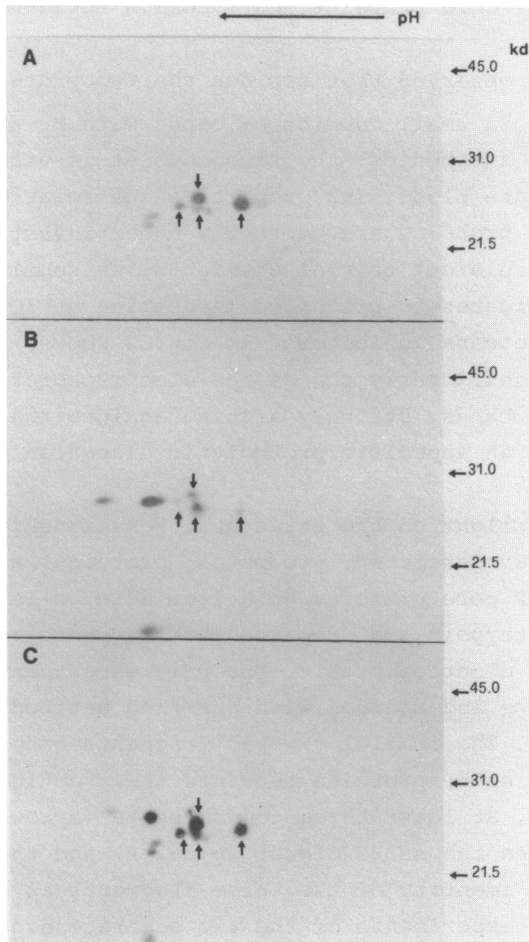tease inhibitors and incubated 2 hours at 30°C, 1.5 mg.

Fig. 3 - Limited digestion with trypsin of ssDBPs and 4OS hnRNP
proteins from HeLa cells and separation of fragments by two-
dimensional electrophoresis. The experiment was performed as
described in Experimental Procedures.
Panel (A): ssDBP; Panel (B): hnRNP core proteins; Panel (C):
ssDBPs plus hnRNP core proteins digested separately and run
together. Arrows indicate common spots. The positions of Mr
markers and of some pI markers are indicated. Kd: Kilodaltons.

12% SDS-polyacrylamide electrophoresis, blotted onto nitrocel-
lulose and stained with the purified antibodies. The results are
shown in Fig. 1, lane 4: the set of bands corresponding to the
described most represented hnRNP polypeptides (6-9) strongly
reacts with the antibodies. These data indicate that mammalian

ssDBPs and hnRNP core proteins share common antigenic determinants.

It should be observed that besides the two protein groups mentioned above, a small doublet of bands with $\underline{Mr}$ around 60,000 reacts with the antibodies; the same doublet is visible also in the total sonicate blots. Its properties and relationships with the other hnRNP proteins are currently under investigation.

In order to rule out trivial artifacts, we submitted crude calf thymus sonicates to prolonged incubation at 30°C in the absence of any protease inhibitors. As Fig. 2 shows, this treatment did not significantly change the electrophoretic pattern of the reactive bands, strongly arguing against the possibility of ssDBPs being an aspecific proteolytic digestion product of hnRNP proteins.

To shed more light on the structural relationships between these two, so far unrelated, groups of proteins, we submitted ssDBPs and hnRNP core proteins, both from HeLa cells, to mild digestion with trypsin and compared the fragments so produced by two-dimensional electrophoresis. The gels were then blotted onto nitrocellulose and stained with purified antibodies, as already described. The initial tryptic fragments generated from both ssDBPs and hnRNP proteins retained their antigenicity and, as Fig. 3 shows, at least three common spots (arrows) can be evidenced between the ssDBP digest (panel A) and the hnRNP (panel B). The virtual identity of the three fragments is confirmed by the comigration experiments of the two separate digests shown in panel C. It is also worth noticing that hnRNP proteins yield two slightly basic fragments, absent in ssDBPs. Mouse hnRNP tryptic fragments of $\underline{Mr}$ between 20,000 and 28,000 have been described also by other authors (14). On the basis of all these data, we conclude that ssDBPs and hnRNP proteins must share extensive structural homology.

DISCUSSION

As to the biological significance of our observations, one intriguing possibility is that the ssDBPs derive from hnRNP proteins by endogenous proteolytic cleavage. Endogenous proteolysis has been also invoked to explain the intrinsic heterogeneity of

ssDBPs (1,4,5), although no direct evidence has so far been obtained. On the other hand, we have been unable to observe any conversion of hnRNP proteins to smaller forms upon prolonged incubation of crude cellular extract in the absence of protease inhibitors (Fig. 2). It is still possible that proteolysis occurs mainly in vivo or that we are observing the effects of a specific controlled pattern of proteolytic cleavage of a large precursor like that described for neuroendocrine peptides (15). Also, we can not rule out minor post-translational modifications, which could still be responsible for at least part of the observed heterogeneity. However, alternative explanations must also be considered, for example, the two groups of proteins could be coded by a family of partially divergent genes or by a single gene with a differential splicing pattern. As we have already mentioned, the two types of proteins share one important property, i.e. strong preferential binding to single-stranded polynucleotides, either ssDNA or RNA. This could account for the structural homologies we have revealed in this work, which in turn point to the existence of common domains in the two groups of proteins and of extensive sequence homology in the corresponding gene(s). Very likely, a definitive answer to these questions will probably be achieved only with the development of monoclonal antibodies and through a detailed analysis of the structure and organization of the gene(s) coding for these proteins. To this purpose, we have already undertaken the screening of a human cDNA library in an expression vector (16), obtaining a few positive clones which are currently under study.

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REFERENCES

1. Herrick,G. and Alberts,B. (1976) J. Biol. Chem. 251,2124-2132.
2. Falaschi,A., Cobianchi,F. and Riva,S. (1980) Trends in Biochemical Sciences 5, 154-157.
3. Riva,S., Clivio,A., Valentini,O. and Cobianchi,F. (1980) Biochem. Biophys. Res. Comm. 96, 1053-1062.
4. Planck,S.R. and Wilson,S.H. (1980) J. Biol. Chem. 255, 11547-11556.
5. Valentini,O., Biamonti,G., Mastromei,G. and Riva,S. (1984) Biochem. Biophys. Acta 782, 147-155.
6. Beyer,A.L., Christensen,M.E., Walker,B.W. and LeStourgeon, W.M. (1977) Cell 11, 127-138.
7. Karn,J., Vidali,G., Boffa,L.C. and Allfrey,V.G. (1977) J. Biol. Chem. 252, 7307-7322.
8. LeStourgeon,W.M., Beyer,A.L., Christensen,M.E., Walker, B.W., Poupore,S.M. and Daniels,L.P. (1978) Cold Spring Harbor Symp. Quant. Biol. 42, 855-897.
9. Martin,T., Billings,P., Pullman,J., Stevens,B. and Kinniburgh,A. (1978) Cold Spring Harbor Symp. Quant. Biol. 42, 899-909.
10. Shapiro,D.J., Taylor,J.M., McKnight,G.S., Palacios,R., Gonzales,C., Kiely,M.L. and Schimke,R.T. (1974) J. Biol. Chem. 249, 3665-3671.
11. Laemmli,U.K. (1970) Nature 227, 680-685.
12. Towbin,H., Staehlin,T. and Gordon,J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350-4354.
13. Douglass,J., Civelli,O., Herbert,E. (1984) Ann. Rev. Biochem. 53, 665-715.
14. Leser,G.P., Escara-Wilke,J. and Martin,T.E. (1984) J. Biol. Chem. 259, 1827-1833.
15. Goldsmith,M.R., Rattner,E.C., Macy,M., Koehler,D., Balikov,S.R. and Bock,S.C. (1979) Anal. Biochem. 99, 33-40.
16. Stanley,K.K. and Luzio,J.P. (1984) Embo J. 3, 1429-1434.