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Characterization of the SS-B (La) antigen in adenovirus-infected and uninfected HeLa cells

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The molecular composition and subcellular localization of the antigens recognized by anti-SS-B (La or Ha) antibodies was investigated. Ten anti-SS-B sera were selected by indirect immunofluorescence and by their immunological identity in counter-immunoelectrophoresis (CIE) with an anti-SS-B reference serum. All sera precipitated virus-associated (VA) RNA from cellular extracts of adenovirus-infected HeLa cells. Earlier results had shown that in adenovirus-infected HeLa cells a cellular 50 000 mol. wt. protein was tightly associated with VA RNA *in situ*. Our present results indicate that this 50 000 protein is the only SS-B antigen present in adenovirus-infected as well as in uninfected cells. A major part (>80%) of the SS-B antigen is present in a readily extractable, soluble form. The rest is found in an insoluble form tightly associated with an internal nuclear structure that is mostly referred to as the nuclear matrix. Both forms are very susceptible to proteolytic degradation resulting in at least two distinct breakdown products of mol. wts. 40 000 and 25 000. The cellular 50 000 polypeptide is present in extracts of various types of cells and tissues, indicating that this antigen is very well conserved during evolution. The association of the 50 000 mol. wt. antigen with host- as well as viral-coded RNA polymerase III products also suggests an important function for this protein in the metabolism of these small RNAs.

Key words: adenovirus/autoimmune antibodies/immunoblotting/SS-B antigen/VA RNA

Introduction

Sera from patients with connective tissue diseases often contain antibodies against cellular components consisting of protein associated with small RNA molecules of ~80-200 nucleotides in length (Lerner and Steitz, 1981). The RNA moiety of some of these ribonucleoprotein particles (RNPs) has already been elucidated. Anti-RNP sera [most commonly obtained from patients with mixed connective tissue disease (MCTD)] include antibodies directed against RNP particles containing one discrete small nuclear RNA called U1 snRNA (Lerner and Steitz, 1979). Anti-Sm sera [mostly obtained from patients with systemic lupus erythematosus (SLE)] recognize particles containing various snRNAs namely U1, U2, U4, U5 and U6 (Lerner and Steitz, 1979). The U-snRNAs precipitated by anti-Sm and anti-RNP antibodies are thought to be RNA polymerase II products (Zieve, 1982). A third class of RNP precipitating antibodies is referred to as anti-SS-B (La or Ha) and is often found in sera from patients with Sjögren's syndrome (Akizuki *et al.*, 1977). These antibodies precipitate a great variety of nuclear RNPs in mouse cells and uninfected HeLa cells (Lerner *et al.*, 1981a). The RNA moiety of these RNPs can be separated on acrylamide gels yielding a highly banded pattern of RNAs ranging in size between 80 and 120 nucleotides (Lerner and Steitz, 1981). These small RNAs are all RNA polymerase III products and some of them have been shown to be precursor molecules (Rinke and Steitz, 1982). From extracts of adenovirus-infected HeLa cells the anti-SS-B sera are able to precipitate a virus-associated RNA (VA RNA) of 158 nucleotides length (Lerner *et al.*, 1981a; Franceour and Mathews, 1982), while from Epstein-Barr virus-infected primate cells the virus-encoded EBER I and II RNAs (180 nucleotides) are precipitated (Lerner *et al.*, 1981b). Both VA and EBER RNA are also RNA polymerase III transcripts (Söderlund *et al.*, 1976).

The protein moiety of the RNPs precipitated by the three categories of sera just described is less well documented. It has been established, however, that it is the protein moiety and not the RNA that carries the antigenic determinants (Lerner and Steitz, 1979). The antigens associated with the U-snRNAs have been described as a group of proteins with mol. wts. between 7000 and 70 000 (Lerner *et al.*, 1981b; Takano *et al.*, 1980; White *et al.*, 1981). Recently some apparently conflicting reports have been published on the nature of the SS-B protein antigens. Franceour and Mathews (1982) found a 45 000 mol. wt. protein to be antigenic while Matter *et al.* (1982) showed that anti-SS-B sera recognize polypeptides of mol. wts. 55 000 and 45 000. Venables *et al.* (1983) also found two antigenic SS-B proteins with mol. wts. of 40 000 and 29 000, and Lieu *et al.* (1982) detected a major 30 000 mol. wt. SS-B antigen. van Eekelen *et al.* (1982a) however, showed that VA RNA in the intact cell is tightly associated with only one protein with a mol. wt. of 50 000. This suggests that, at least in adenovirus-infected cells, this protein is the antigen recognized by anti-SS-B antibodies able to precipitate VA RNA. Here we show that the SS-B antigen both in adenovirus-infected and in uninfected HeLa cells is a cellular 50 000 mol. wt. protein that is very susceptible to proteolytic degradation. This instability might be the reason for the apparently conflicting results obtained so far.

Results

Specificity of the anti-SS-B sera

To define patient sera as anti-SS-B, they had to fulfill each of the following four criteria. (I) Positive nuclear staining in indirect immunofluorescence on HeLa monolayer cells. (II) Anti-SS-B positive reaction in a counter-immunoelectrophoresis (CIE) assay, i.e., (a) RNase-insensitive, trypsin-sensitive precipitation reaction with a rabbit thymus extract, mostly referred to as ENA (extractable nuclear antigen) as substrate (not shown). (b) Immunological identity with a reference anti-SS-B serum and no (partial) identity with RNP or Sm reference sera (not shown). (III) Precipitation of VA

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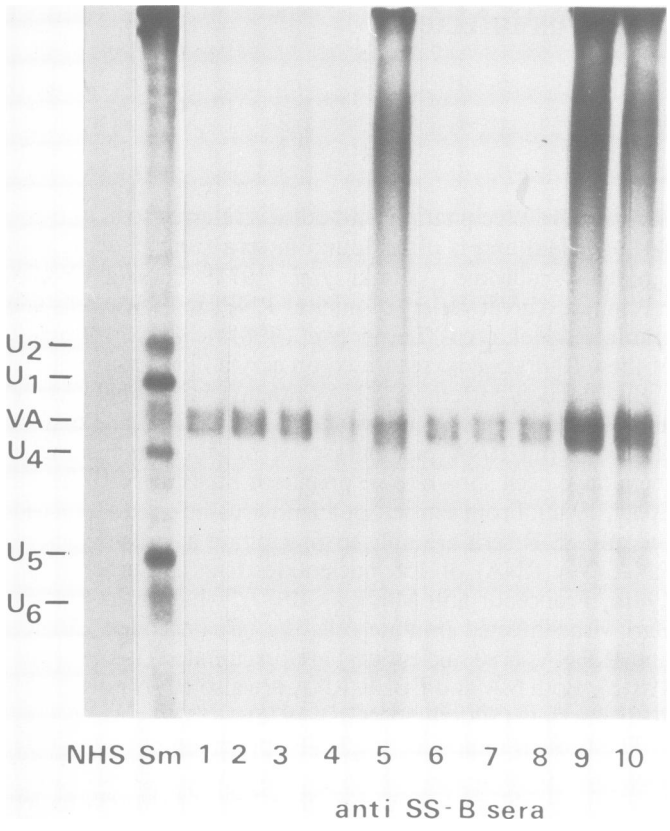

Fig. 1. Characterization of anti-SS-B sera by RNA precipitation. For the precipitation with normal human serum (NHS) and anti-SS-B sera, an extract from adenovirus-infected HeLa cells was used as source of antigens. U-snRNAs were precipitated from an uninfected HeLa cell extract (see Materials and methods). The figure shows a fluorogram of immunoprecipitated [^{3}H]uridine-labeled RNAs using NHS, reference anti-Sm serum, and 10 anti-SS-B sera selected by immunofluorescence and CIE as described in the text.

RNA from adenovirus-infected HeLa cells (Figure 1). (IV) No U-snRNAs precipitated from uninfected HeLa cells (not shown).

Ten sera, at first selected via criteria I and II, were subsequently shown to fulfill the other criteria as well. These anti-SS-B sera were obtained from patients mostly diagnosed as SLE, using the 1982 revised criteria for the classification of SLE, (Tan *et al.*, 1982), or as secondary Sjögren's syndrome (Table I). For the diagnosis of Sjögren's syndrome the patient had to have at least two of the following clinical features of the sicca complex: xerophthalmia, xerostomia or salivary gland enlargement.

Cellular localization of the SS-B antigen using the indirect immunofluorescence technique

It is known that the SS-B antigen(s) are primarily localized in the nucleus when tested by indirect immunofluorescence on various types of cells (Tan, 1982). In HeLa cells, a speckled nuclear fluorescent pattern is observed with slight cytoplasmic staining depending on the particular serum tested. Since anti-SS-B sera are able to precipitate VA RNA from a cytoplasmic extract of adenovirus-infected cells, we investigated whether SS-B specific antigens could be detected in the cytoplasm of HeLa cells late after infection with adenovirus. Indirect immunofluorescence studies were performed on HeLa S3 monolayers 14 h after infection. Control experiments using anti-total adenovirus protein antibodies raised in rabbits,

Table I. Clinical and serological features of 10 patients with anti-SS-B antibodies

Patient number	Sex	Age at testing	Diagnosis[a]	Anti-DNA antibodies
1	M	33	UCTD	–
2	F	57	SS/arthralgia	–
3	M	36	SLE	–
4	F	45	SS/SLE	–
5	F	42	SLE	+
6	F	60	SS/RA	–
7	F	82	RA	–
8	F	41	UCTD	–
9	F	31	SLE	+
10	F	20	SS/RA	–

[a]UCTD, Undifferentiated connective tissue disease; RA, rheumatoid arthritis; SS/SLE, Sjögren's syndrome with systemic lupus erythematosus; SS/RA, rheumatoid arthritis with sicca syndrome.

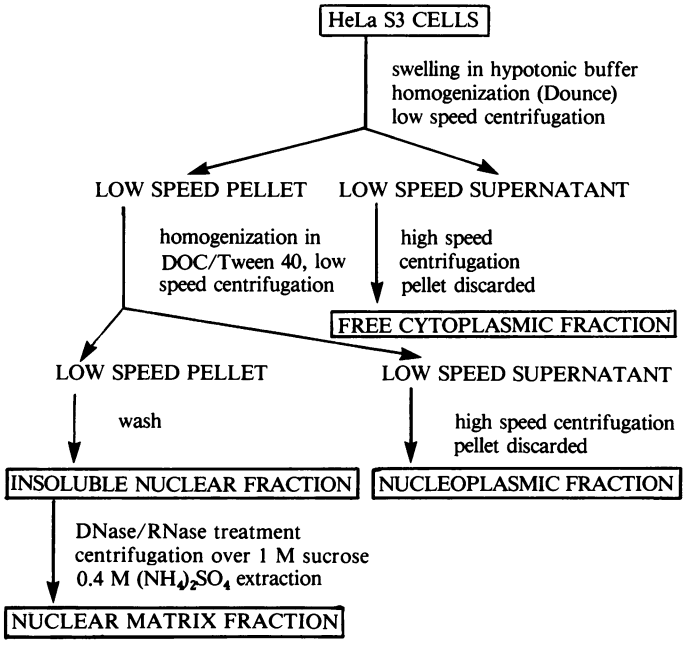

Fig. 2. Isolation scheme of HeLa cell protein fractions.

showed that 95–100% of the HeLa cells were infected. Nevertheless, the fluorescence of the SS-B antigen in these infected cells is still predominantly nuclear as it is in uninfected cells (data not shown). With almost all anti-SS-B sera some discrete fine speckles in the cytoplasm of infected cells could be observed. These speckles were not specific for anti-SS-B sera since several normal human control sera showed exactly the same phenomenon. It is possible that this cytoplasmic staining is due to the fact that almost every (human) serum contains, to some extent, antibodies directed against adenovirus proteins, and the fine speckles, therefore, might represent an accumulation of these antigens at specific locations in the cytoplasm of infected HeLa cells. It should be emphasized that the nuclear fluorescence was typical for the anti-SS-B sera; none of the human control sera showed nuclear fluorescence neither in adenovirus-infected nor in uninfected HeLa cells.

These indirect immunofluorescence experiments do not exclude the possibility that the SS-B antigen is located in the

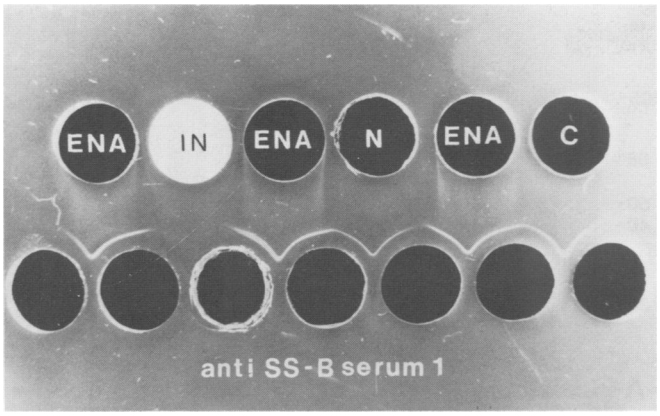

Fig. 3. Detection of SS-B antigens in HeLa cell protein fractions and ENA by counter-immunoelectrophoresis. The cell fractions were prepared as depicted in Figure 2. IN: insoluble nuclear fraction; N: nucleoplasmic fraction; C: cytoplasmic fraction.

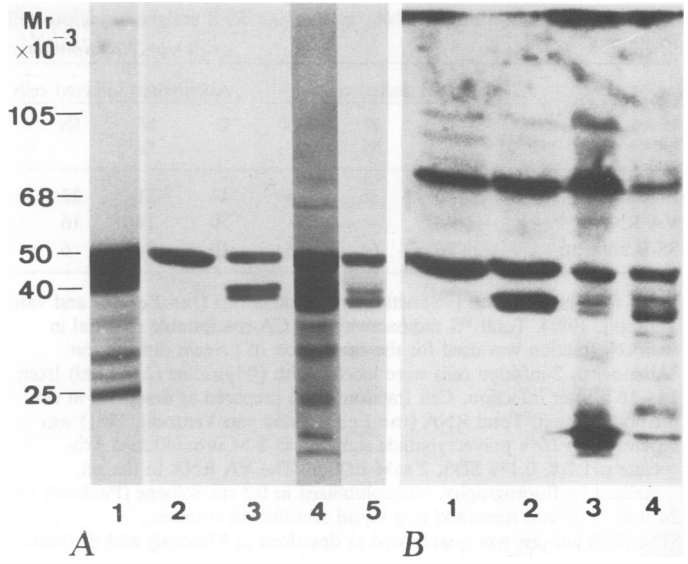

Fig. 4. Detection of SS-B antigens by immunoblotting. **(A)** ENA and uninfected HeLa cell protein fractions were isolated as described in Materials and methods and separated into their individual polypeptide constituents by SDS-PAGE. Subsequently the gel was blotted electrophoretically onto nitrocellulose and probed with anti-SS-B serum no. 1. Immunoreactive species were detected using ^{125}I-labeled protein A. Protein fractions used were: **lane 1**, ENA; **lane 2**, free cytoplasmic fraction; **lane 3**, nucleoplasmic fraction; **lane 4**, insoluble nuclear fraction; **lane 5**, nuclear matrix fraction. **(B)** Detection of antigens in adenovirus-infected HeLa cell protein fractions. **Lane 1**, free cytoplasmic fraction; **lane 2**, nucleoplasmic fraction; **lane 3**, insoluble nuclear fraction; **lane 4**, nuclear matrix fraction.

cytoplasm *in vivo*. After serum incubation of the alcohol/acetone fixed slides the unbound immunoglobulins and other serum proteins were removed from the preparation by several washes with barbitone buffer. Even after high speed centrifugation (15 min, 8000 *g*) the collected washing buffer contained SS-B antigens in very easily detectable amounts, when tested by immunoblotting. This suggests that at least part of the SS-B antigen may not be detected by the indirect immunofluorescence technique since it can be washed out of the fixed monolayer cells.

Detection of SS-B antigens in HeLa cell fractions by CIE and immunoblotting

For the localization of antigens a cell fractionation procedure was used that resulted in a free cytoplasmic, a nucleoplasmic and an insoluble nuclear fraction (Figure 2). The free cytoplasmic fraction probably also contains some readily soluble components of the nucleoplasm, due to the Dounce homogenization procedure in hypotonic buffer. The nucleoplasmic fraction certainly contains cytoskeleton components solubilized during the detergent treatment. Finally, the insoluble nuclear fraction contains chromatin and nuclear RNA protein complexes associated with the internal nuclear ultrastructure generally referred to as nuclear matrix.

These three cellular fractions were tested by CIE with all anti-SS-B sera. As is shown in Figure 3, the free cytoplasmic and nucleoplasmic fractions apparently contain (an) identical antigen(s). They also show identical precipitation lines with antigens extracted from rabbit thymus powder (ENA). No precipitation lines could be observed with the insoluble nuclear fraction, indicating that this fraction does not contain sufficient amounts of soluble antigens any more. No different specificities could be detected between sera with and without anti-DNA antibodies. All sera gave a CIE precipitation pattern identical to the one shown in Figure 3 (serum no. 1).

For the detection of both soluble and insoluble antigens the immunoblotting technique is very well suited. After solubilization of the antigen-containing fractions in 2% SDS-containing sample buffer, antigens can be separated according to their mol. wt. by polyacrylamide gel electrophoresis (PAGE), blotted onto nitrocellulose, and used as a substrate for the immunoreaction (see Materials and methods). For the immunoblotting experiments a nuclear matrix preparation was also used as a source of antigen(s). This fraction was prepared from the insoluble nuclear fraction by subsequent DNase/RNase treatment and sucrose – high salt extraction as described in Materials and methods. After separation by PAGE, the four cellular fractions and ENA all showed a specific polypeptide pattern. After immunoblotting, in all these fractions the same 50 000 mol. wt. antigen was recognized by SS-B serum no. 1 (Figure 4A). In the cytoplasmic fraction this 50 000 polypeptide seems to be the only antigen, in contrast to the other fractions and ENA, which appeared also to contain an antigenic polypeptide of mol. wt. 40 000. This antigen will be shown to be an *in vitro* breakdown product of the 50 000 protein (see below).

Since all sera precipitate a viral-coded (VA) RNA from extracts of adenovirus-infected cells, we investigated whether SS-B specific proteins of viral origin exist. The fractionation and immunoblotting experiments were repeated with HeLa cells 4 and 16 h after infection with adenovirus serotype 2. The polypeptide pattern and immunoblotting results obtained with the 4 h infected cells were identical with those obtained with the uninfected cells (not shown). In the late infected cells, however, some additional reactive polypeptides could be observed (Figure 4B). These major viral antigens have approximate mol. wts. of 80 000 and 105 000 and therefore could be identical to the viral hexon and penton capsid proteins. The antibodies against these viral antigens were obviously not characteristic for anti-SS-B sera since these antigens were also recognized by antibodies present in normal human control sera (from 20 normal sera tested, 18 contained antibodies against the 80 000 protein and three sera showed anti-anti-105 000 activity). We therefore conclude that also in adenovirus-infected cells a host-coded 50 000 mol. wt. protein is the major antigen recognized by anti-SS-B antibodies.

Table II. Distribution of VA RNA, protein and SS-B antigen in various cell fractions

	Uninfected cells			Adenovirus-infected cells		
	C[d]	N	IN	C	N	IN
		%			%	
Protein[a]	40	36	24	47	31	22
VA RNA[b]	–	–	–	70	14	16
SS-B antigen[c]	20	64	16	10	84	6

[a]Cells were labeled with [^{35}S]methionine as described (van Eekelen and van Venrooij, 1981). Total ^{35}S radioactivity in TCA-precipitable material in each cell fraction was used for the calculation of protein distribution.
[b]Adenovirus 2-infected cells were labeled with [^{3}H]uridine (2 μCi/ml) from 14–16 h after infection. Cell fractions were prepared as described in the methods section. Total RNA (van Eekelen and van Venrooij, 1981) was separated on 10% polyacrylamide slab gels in 8 M urea/40 mM Tris-acetate pH 7.8, 0.1% SDS, 2 mM EDTA. The VA RNA in the gel, visualized by fluorography, was solubilized in 0.5 ml Soluene (Packard) for 24 h at 55°C and measured in a liquid scintillation counter.
[c]The SS-B antigen was quantitated as described in Materials and methods.
[d]C = free cytoplasmic fraction; N = nucleoplasmic fraction; IN = insoluble nuclear fraction.

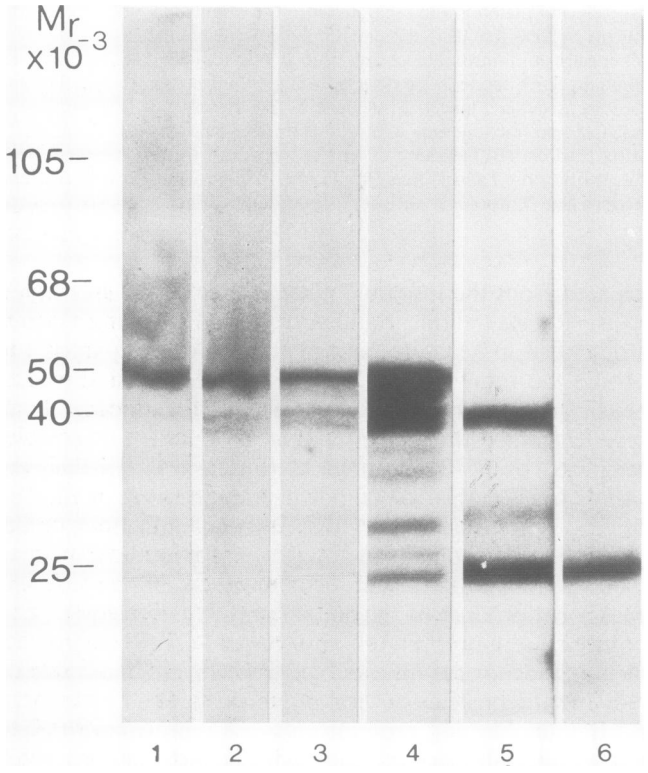

Fig. 5. *In vitro* degradation of the SS-B antigen from rabbit thymus. Rabbit thymus acetone powder (Pel Freez) was extracted at 4°C with PBS containing 0.5 mM PMSC to inhibit proteolytic activities. Aliquots were removed at different time intervals and centrifuged for 5 min at 10 000 *g* at 4°C. Both pellet and supernatant were analysed after SDS gel electrophoresis by immunoblotting. **Lane 1**, total rabbit acetone thymus powder; **lane 2**, pellet after 10 min extraction; **lane 3**, supernatant after 10 min extraction; **lane 4**, supernatant after 4 h extraction (ENA); **lane 5**, ENA after 2 month storage at −20°C; **lane 6**, ENA after incubation at 37°C overnight.

Most of the SS-B antigen is present in the nucleoplasmic fraction

To obtain more quantitative information about the distribution of the SS-B antigen in the various cell fractions, a semi-quantitative immunoblotting analysis (see Materials and methods) was carried out. The results, obtained with various batches of cells, clearly show that the majority of the SS-B antigen in uninfected as well as in infected cells was present in the nucleoplasmic fraction (Table II). These results indicate that the bulk of the SS-B antigen is released from the nucleus by the DOC-Tween 40 treatment (see Materials and methods). Using adenovirus-infected cells we also quantitated the relative amounts of VA RNA in each cell fraction. About 70% of the VA RNA was found in the free cytoplasmic fraction which contains only 8–12% of the total cellular content of SS-B antigen. Immunoprecipitation studies have shown that VA RNA is almost quantitatively associated with the SS-B antigen (Lerner *et al.*, 1981a; our unpublished observations). In other words, ~8–12% of the SS-B antigen in the free cytoplasmic fraction is probably sufficient to complex 70% of the VA RNA (Table II). Since about eight times as much SS-B antigen is present in the nucleoplasmic fraction, this strongly suggests that at least part of the SS-B antigen is present in the nucleus in an uncomplexed, i.e., a not RNA-associated form.

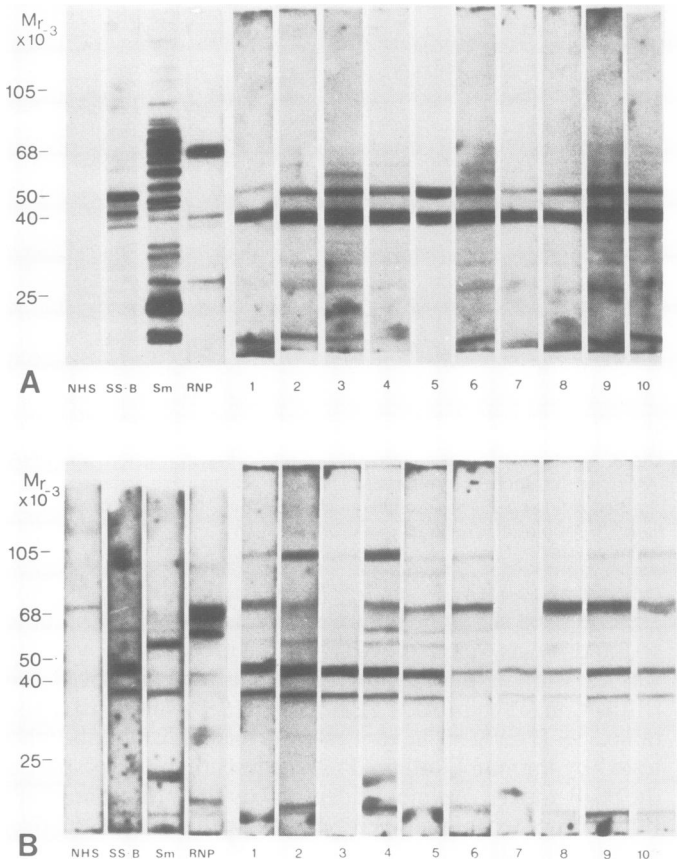

Fig. 6. Analysis by immunoblotting of anti-SS-B sera and reference sera. **(A)** The protein blot from one gel loaded over the entire width with a nuclear protein fraction of uninfected HeLa cells, was cut into strips and probed with various anti-SS-B sera **(lanes 1–10)** and reference anti-SS-B, anti-RNP and anti-Sm sera. NHS: normal human serum. **(B)** as **A**, but now a nucleoplasmic extract from adenovirus-infected cells was used as source of antigens.

In vitro breakdown of the SS-B antigens

Because all sera recognized always both the 50 000 and 40 000 polypeptides with about the same relative efficiency, the possibility that they were related to each other was investigated. When commercially available acetone extracted rabbit thymus powder was sonicated, solubilized in 2% SDS-

containing sample buffer and tested by immunoblotting, only the 50 000 antigen was detectable (Figure 5, lane 1). ENA, prepared by extraction of this thymus powder with phosphate buffered saline (PBS), contained additional 40 000 and 25 000 antigens (Figure 5, lane 4) although extraction was performed under sterile and RNase-free conditions with 0.5 mM phenylmethylsulphonyl chloride (PMSC) as an inhibitor of proteolysis added to the extraction buffer. In fact, this breakdown occurs very rapidly, since a 10 min extraction already induces the presence of the 40 000 antigen (Figure 5, lane 3). The pellet remaining after 10 min extraction was washed twice with PBS but nevertheless still contained the 40 000 as well as the 50 000 antigen (Figure 5, lane 2). The breakdown of the SS-B antigen into a 25 000 polypeptide occurs at a somewhat slower rate, but is completed after incubation of the ENA overnight at 37°C (Figure 5, lanes 5 and 6).

It should be pointed out that the 40 000 and 25 000 polypeptides are discrete breakdown products carrying the SS-B specific determinant(s). Some other breakdown products might also exist which do not carry an antigenic determinant and are therefore not recognized in immunoblotting experiments.

All anti-SS-B sera recognize the same protein antigen

From earlier work we know that a particular type of human auto-antibodies (for example anti-RNP or anti-Sm), although precipitating a specific set of small RNAs, may recognize a greatly varying set of proteins (Habets *et al.*, 1983). We therefore investigated whether such a diversity also existed among the anti-SS-B sera. As is shown in Figure 6A, all anti-SS-B sera, including the SS-B reference serum, only recognized the 50 000 mol. wt. antigen and its breakdown products in an insoluble nuclear fraction from uninfected HeLa cells.

This same cellular 50 000 antigen was also recognized by all these sera in adenovirus-infected cells (Figure 6B). Such a specific recognition of only one antigen by a class of human auto-immune sera is in contrast with the findings reported for anti-RNP and anti-Sm sera. With such sera, mostly several antigens are detected by the immunoblotting method (Habets *et al.*, 1983). The immunoblot patterns of these anti-RNP and anti-Sm sera also depend on the cellular substrate offered (Figure 6A,B). Furthermore, there is also a great variation in antigen patterns within one class (Habets *et al.*, 1983), a phenomenon that is also not observed with anti-SS-B sera. As discussed above for serum no. 1 (Figure 4), the other SS-B sera also have rather high levels of antibodies against adenovirus-coded proteins. Sera 2 and 4 show a strong reaction against the 105 000 mol. wt. protein, and eight out of 10 anti-SS-B sera recognize the 80 000 mol. wt. adenoprotein. As expected, all sera (10 anti-SS-B, the three reference sera and 20 tested normal human control sera) reacted positively in an enzyme-linked immunosorbent assay that measured the anti-adenovirus protein antibody titer (A.van Loon, personal communication).

The RNA binding 50 000 mol. wt. SS-B antigen is evolutionarily highly conserved

Various types of RNA molecules can be precipitated by anti-SS-B sera when extracts from either uninfected mouse cells or human cells are used as a substrate. For this reason it was concluded that the SS-B RNPs were evolutionarily not as highly conserved as, for example, the U-snRNPs (Lerner *et al.*, 1981a). Our results show that in rabbit thymus extracts and human HeLa cells only one protein antigen is recognized by anti-SS-B sera (Figure 4). This 50 000 mol. wt. protein is also the only antigen recognized by anti-SS-B sera in other types of cells and tissues like rat liver, hamster lens, human lymphoblasts in culture (Rosenfeld *et al.*, 1977) and cultured hepatoma cells (Alexander *et al.*, 1976) (data not shown). Since in all these types of cells the breakdown products of the 50 000 antigen are also identical, it seems that the protein part of the SS-B antigen is very highly conserved.

Discussion

We have shown that the SS-B antigen is a cellular protein with a mol. wt. of 50 000. This protein is the only SS-B specific antigen in uninfected as well as in adenovirus-infected cells. Several RNA species, in particular precursors of RNA polymerase III transcripts, can be precipitated by anti-SS-B sera by virtue of their association with the SS-B specific antigen. For this reason, we assume that the 50 000 mol. wt. antigen is associated with all these RNA species. This assumption is supported by the fact that VA RNA specifically recognizes a 50 000 mol. wt. protein in a cytoplasmic extract immobilized on nitrocellulose sheets (van Eekelen *et al.*, 1982a). Furthermore, VA RNA has been shown to be associated with a 50 000 mol. wt. protein in the intact cell after RNA-protein cross-linking *in vivo* by u.v. irradiation of adenovirus-infected HeLa cells (van Eekelen *et al.*, 1982a). All these results strongly point to the fact that SS-B specific RNAs (including EBER and the RNA polymerase III precursors), at least for some time during their life cycle are associated *in vivo* with the 50 000 protein.

The 50 000 SS-B antigen is very susceptible to proteolytic degradation. Even after 10 min extraction of rabbit thymus powder, a 40 000 degradation product, derived from the 50 000 antigen, can be detected in the ENA extract as well as in the residual pellet (Figure 5). After a 4 h extraction, both the 40 000 and a 25 000 degradation product are detectable. These degradation products are not found in cytoplasmic extracts of HeLa cells except when detergents are used. We assume that the detergent treatment releases proteolytic activities by permeabilizing or dissolving cytoplasmic structures like lysosomes. In rabbit thymus powder, the source of ENA, such structures are already severely damaged and permeabilized by the acetone extraction and proteolytic activities might therefore have been released. The rapid rate of degradation of the SS-B antigen explains why it has been described as a protein with mol. wt. varying from 29 000 to 45 000 (Franceour and Mathews, 1982; Matter *et al.*, 1982; Venables *et al.*, 1983; Lieu *et al.*, 1982).

The quantitation of the SS-B antigen in the various cell fractions showed that most of the SS-B antigen is present in the nucleoplasmic fraction. Because immunofluorescence experiments clearly indicate that the SS-B antigen is located in the nucleus, we conclude that the majority of the SS-B antigen is readily extracted from the nucleus by simple detergent treatment. Nevertheless, since the detergent-extracted nuclei, even after repeated washing, still contain $\sim 6-16\%$ of the total cellular amount of SS-B antigen (Table II) we must also conclude that part of the SS-B antigen is present in the nucleus in a rather insoluble form. Removal of the chromatin and most of the nuclear RNA by nuclease treatment and high salt extraction does not remove these residual SS-B antigens. It is therefore probable that this portion of the SS-B antigen is

associated with the nuclear matrix (compare Figure 4), a nuclear ultrastructure that is involved in the processing of various RNA transcripts like pre-5S RNA and pre-mRNA sequences (Ciejek *et al.*, 1982; Mariman *et al.*, 1982; Ross *et al.*, 1982). The free cytoplasmic fraction of uninfected as well as infected cells also contains 10–20% of the SS-B antigen. It might be possible that these antigens are extracted from the nucleus during homogenization of the cell suspension in hypotonic medium. However, since the SS-B antigen in adenovirus-infected cells is associated *in vivo* with VA RNA (Lerner *et al.*, 1981a; van Eekelen *et al.*, 1982a) and VA RNA has a cytoplasmic localization (Table II) and most probably a cytoplasmic function as well (Thimmappaya *et al.*, 1982), one would expect some of the SS-B antigen to be present in the cytoplasm of infected cells. Indirect immunofluorescence experiments that point to an exclusive nuclear localization do not contradict such a statement because we have observed that soluble SS-B antigens are easily extracted from the acetone-fixed cells during the subsequent washing with buffer solutions. It is therefore possible that cytoplasm-localized SS-B antigens not associated with structural cell components are not detected by the indirect immunofluorescence assay because of their extreme solubility.

Another point of interest is the fact that the amount of SS-B antigen in the cytoplasmic fraction of adenovirus-infected cells (8–12% of the total cellular amount) is sufficient to complex ~70% of the cellular VA RNA (Lerner *et al.*, 1981a; our unpublished observations). The nucleoplasmic fraction, however, contains about eight times as much SS-B antigen and only a small part of the VA RNA. These data suggest strongly that part of the SS-B antigen in the nucleus of adenovirus-infected cells is present in an uncomplexed, not RNA associated form.

Materials and methods

Sera

Sera from patients [mostly with SLE or Sjögren's syndrome associated with rheumatoid arthritis (RA) or SLE] were obtained from the St. Radboud Hospital in Nijmegen. They were first screened for anti-nuclear antibodies (ANA) by the indirect immunofluorescence test on HeLa cell monolayers essentially as described (van Eekelen *et al.*, 1982b).

All ANA-containing sera were then screened for anti-SS-B antibodies by CIE using a saline extract (ENA) of rabbit thymus powder (Pel Freez, Rogers, AR) as antigen source (Kurata and Tan, 1976). The sera which showed an RNase-insensitive, trypsin-sensitive precipitation reaction, were tested for identity with anti-SS-B, anti-Sm and anti-RNP reference sera. Using these criteria, we selected ~10 anti-SS-B sera with which this study was performed. All these sera were anti-Sm and anti-RNP negative. Only two contained anti-DNA antibodies as tested by the *Crithidia* assay (Aarden *et al.*, 1975).

Reference sera were obtained from the Centers for Disease Control, Atlanta, GA.

Culturing and infection of cells

HeLa S3 cells were grown in suspension and monolayer cultures as described (van Eekelen and van Venrooij, 1981). They were shown not to contain Epstein-Barr virus and regularly performed mycoplasma tests were always negative.

HeLa S3 cells were infected with purified adenovirus serotype 2 as described (van Eekelen *et al.*, 1981). The percentage of infected cells, 14 h after infection (mostly 95–100%), was determined by indirect immunofluorescence with an anti-total adenovirus-protein serum [kindly provided by F.Asselbergs (Asselbergs *et al.*, 1983)].

RNA immunoprecipitation

HeLa S3 suspension culture cells were labeled with 2 μCi/ml [^{3}H]uridine at a density of 0.5 x 10^6 cells/ml for 24 h. Nuclear supernatants were prepared by the following procedure carried out at 0–4°C. Cells were harvested at 800 *g* for 5 min on frozen NKM [130 mM NaCl, 5 mM KCl, 1.5 mM $Mg(Ac)_2$], washed once with NKM, pelleted and resuspended in reticulocyte suspension buffer (RSB, 10 mM Tris (pH 7.4), 1.5 mM $MgCl_2$, 10 mM NaCl, 0.5 mM PMSC). Cells were then broken by 15 strokes in a Dounce homogenizer and the crude nuclei were pelleted at 800 *g* (5 min). The crude nuclei were resuspended in RSB and sonicated 2 x 15 s with a Branson sonifier at setting 2. After centrifugation (5400 *g*, 5 min), this nuclear supernatant was used as source of antigens for the immunoprecipitation of U-snRNPs.

Extraction of antigens from adenovirus-infected cells was performed slightly differently because most of the VA RNA is present in the cytoplasm. Labeling from 14–16 h after infection and harvesting procedures of cells were identical but then the cell pellet was resuspended in RSB. A mixture of sodium deoxycholate (DOC) and Tween 40 was added (final concentrations 0.5% and 1%, respectively), cells were broken by brief shaking on a vortex shaker and pelleted at 2000 *g* for 5 min. The supernatant was used for the immunoprecipitation of VA RNA.

In a typical RNA precipitation experiment, 15 μl of IgG selected by protein A-Sepharose affinity chromatography was incubated at 0°C for 20 min with the nuclear or cellular extract from 50 x 10^6 cells. Then 200 μl 10% protein A-Sepharose in PBS (3 mM NaH_2PO_4, 7 mM Na_2HPO_4, 0.9% NaCl, pH 7.6) was added and the mixture was left on ice for 20 min. Immune complexes were isolated by centrifugation, and washed 5 times with PBS. The precipitated RNAs were extracted with phenol and analysed on 10% polyacrylamide slab gels in 8 M urea/40 mM Tris-acetate pH 7.8/0.1% SDS/2 mM EDTA. After electrophoresis overnight, gels were prepared for fluorography.

Preparation of HeLa cell fractions

The fractionation procedure (Figure 2) was carried out at 0–4°C unless otherwise stated. A hypotonic swelling method was used to prepare cytoplasmic extracts. Cells were harvested on frozen NKM, washed with NKM, resuspended in hypotonic buffer (RSB) and left on ice for 5 min. The cells were then disrupted in an all glass Dounce homogenizer (Type B, seven strokes) followed by low speed (1000 *g*, 5 min) centrifugation to pellet the nuclei. The supernatant was clarified by high speed centrifugation (8000 *g*, 15 min) and is referred to hereafter as free cytoplasmic extract. The crude nuclei were resuspended in RSB and a DOC/Tween 40 mixture (final concentrations 0.5% and 1%, respectively) was added followed by homogenization with 10 strokes of a motor-driven Teflon pestle in a Potter-Elvehjem tissue homogenizer. The homogenate was centrifuged at low speed, the supernatant was then clarified by high speed centrifugation (8000 *g*, 15 min) and is referred to hereafter as nucleoplasmic fraction. The low speed pellet was washed twice with RSB, resuspended in RSB and is further referred to as insoluble nuclear fraction.

In some experiments this insoluble nuclear fraction was subfractionated into a chromatin fraction and a nuclear matrix fraction (Figure 2). For this purpose the insoluble nuclear fraction was resuspended in HRSB [110 mM NaCl, 10 mM Tris (pH 7.4), 1.5 mM $MgCl_2$ and 0.5 mM PMSC] at a density of 10^8 nuclei/ml and treated with DNase I and RNase A (500 μg/ml and 100 μg/ml, respectively) for 1 h at 20°C. Fragmented chromatin and RNP complexes were removed by sedimentation through a 1 M sucrose layer in HRSB (2000 *g*, 10 min) followed by an extraction with 0.4 M $(NH_4)_2SO_4$. The pellet was washed and resuspended in RSB and is referred to hereafter as nuclear matrix fraction. Morphological and biochemical characteristics of nuclear matrices isolated in this way have been described (van Eekelen *et al.*, 1982b).

Gel electrophoresis and protein blotting

Samples for PAGE in SDS were prepared by dissolving the protein fraction in sample buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.1 M Tris-HCl pH 6.8). To ensure complete dissociation of the protein complexes, the samples were heated for 3 min at 100°C followed by gel electrophoresis in SDS on 10% polyacrylamide slab gels (1 mm thick, 10 x 16 cm). In most experiments proteins were loaded over the entire width of the gel (1.5 mg protein per gel) and separated according to their mol. wt. essentially as described (Laemmli, 1970). After a 4 h run at 20 mA per gel, replicas of the gels were made on nitrocellulose (i.e., blotting) by transferring the proteins electrophoretically using a Bio-Rad trans-blot cell. Transfer was performed overnight at room temperature and at 60 V/0.3 A in 192 mM glycine, 25 mM Tris pH 8.3 and 20% methanol. After transfer the blots were dried and stored at room temperature.

Detection of antigens

The protein blots were cut from top to bottom into strips of ~7 mm and treated with pre-incubation buffer [3% bovine serum albumin (BSA), 350 mM NaCl, 10 mM Tris.HCl pH 7.6, 0.5 mM PMSC] for 3 h at 20°C to saturate additional protein binding sites on the nitrocellulose. Incubation with diluted serum (mostly 1:50) was performed overnight in buffer 1 (0.3% BSA, 150 mM NaCl, 10 mM Tris.HCl pH 7.6, 0.1 mM PMSC, 1% Triton X-100, 0.5% DOC and 0.1% SDS). After extensive washing with buffer 1 (3 x

10 min) IgG immune complexes were detected by incubating the blots for 2 h with ^{125}I-labeled protein A (sp. act. 1 mCi/mg) in buffer 1 (2 μCi in 10 ml) then washed again with buffer 1 (3 x 10 min) and water (3 x 10 min), dried under a lamp and exposed to X-ray film for 2 – 16 h at – 70°C using Ilford intensifying screens.

In some blotting experiments, ^{125}I-labeled anti-human IgG was used instead of protein A. The results were the same (not shown).

Quantitation of the SS-B antigen

For the quantitation of the SS-B antigen in the various cell fractions, serial dilutions of the antigen-containing fractions were subjected to PAGE and subsequently blotted onto nitrocellulose. For these experiments we used the horizontal protein blotting procedure as described by Vaessen *et al.* (1981), that resulted in a >90% efficient transfer of the proteins. (This percentage was calculated from transfers carried out with [^{35}S]methionine-labeled cell fractions). Protein blots containing serial dilutions of the various cell fractions were incubated with excess anti-SS-B serum for 2 h and then washed with PBS containing 0.5% Triton X100. Immune complexes were detected either with horseradish peroxidase-labeled anti-human IgG (Nordic, Tilburg, The Netherlands) or with ^{125}I-labeled protein A as described (Habets *et al.*, 1983). Results were read in several ways. In the case of the peroxidase-labeled second antibody the highest dilution that gave a signal above background was determined visually. In the case of the ^{125}I-labeled protein A the autoradiograms were scanned and quantitated as described (van Eekelen *et al.*, 1982c). Both ways of reading gave identical results.

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Note added in proof

Recent results of the group of S.O.Hoch and of J.Stefano (Abstracts presented at the Cold Spring Harbor meeting on RNA processing, May 1983) also showed that a polypeptide of 50 000 mol. wt. is the only SS-B specific antigen.