MOLECULAR AND CELLULAR BIOLOGY, July 1987, p. 2588–2591
0270-7306/87/072588-04$02.00/0

Vol. 7, No. 7

NOTES

The Small Nuclear Ribonucleoprotein SS-B/La Binds RNA with a Conserved Protease-Resistant Domain of 28 Kilodaltons†

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Received 8 December 1986/Accepted 30 March 1987

SS-B/La is a nuclear protein of 48 kilodaltons with two structural domains of M_r 28,000 and M_r 23,000 generated by proteolytic cleavage. UV irradiation was used to cross-link preexisting intracellular La-RNA complexes. Subsequent protease digestion and diagonal gel electrophoresis showed that the RNA-binding site resided in the nonphosphorylated, methionine-rich 28-kilodalton domain.

SS-B/La is a conserved nuclear protein found in many mammalian species (11, 23) and is transiently associated with a number of small RNA species in the form of ribonucleoprotein particles (16). The SS-B/La-associated RNA species include many RNA polymerase III transcripts (3, 9, 19, 21–25, 29). In addition, it has been reported that SS-B/La also binds to U1 RNA, a polymerase II transcript (18), and to the vesicular stomatitis virus leader RNA (31). It appears that the 3′-oligouridylate tail of these small RNAs is required for interaction with the SS-B/La protein (19, 22, 25), but the nature of the RNA-binding region has not been examined.

SS-B/La was initially recognized as a target antigen of autoantibodies found in sera of patients with systemic lupus erythematosus and Sjögren's syndrome (1, 20, 26). It has recently been shown that there are two protease-resistant domains (X and Y) in SS-B/La from HeLa cells (4). Domain X is a methionine-rich, nonphosphorylated 28-kilodalton (kDa) polypeptide. Domain Y (23 kDa) contains little if any methionine but all the detectable phosphorylated amino acids (4). Both structural domains contain leucine residues (Fig. 1). Monolayer HeLa cells were labeled with [^{3}H]leucine (RPI Corp.) at 50 μCi/ml in leucine-free medium (Flow Laboratories) supplemented with 2% fetal calf serum and grown for 16 h. Cells were extracted in 1% Empigen BB (Albright & Wilson) in phosphate-buffered saline (5) and were freed of cell nuclei and debris by centrifugation at 12,000 × g for 15 min. Samples of cell extracts were first digested with various concentrations of S.a.V8 protease for 30 min at 37°C and then immunoprecipitated with anti-SS-B/La serum Ze, the Centers for Disease Control reference serum (27). This anti-SS-B/La serum was known to contain antibodies which were reactive with both domains X and Y (4). Protein A-Sepharose-facilitated immunoprecipitation was performed as described elsewhere (5, 9). The control (Fig. 1, lane 1) was incubated at 37°C for 30 min without protease and showed a single band of 48 kDa. At a 10-μg/ml protease concentration (lane 2), fragments X′, X, and Y′ of 29, 28, and 24 kDa, respectively, were detected. At higher concentrations of protease (lanes 3 to 5), smaller fragments of 17 and 20 kDa were detected. These 17- and 20-kDa fragments were phosphorylated, and they were probably derived from the Y/Y′ fragments (Fig. 2; see also Fig. 4 and 5). The X fragments were more resistant to digestion over a range of 1- to 80-fold protease concentrations and, therefore, were more stable than the Y fragments in terms of protease resistance.

RNA association with SS-B/La domains. By using the same experimental conditions of S.a.V8 digestion but with $^{32}P_i$ label (New England Nuclear Research Products) at 100 μCi/ml, precursor tRNAs were precipitated (Fig. 2B, lanes 3 to 5) when the native 48-kDa protein was no longer detected (Fig. 2A, lanes 3 to 5); this procedure showed that RNA

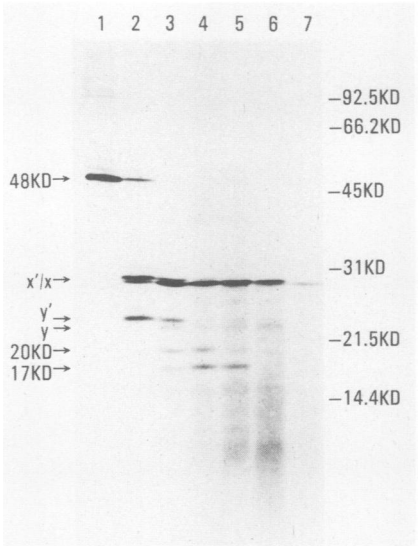

FIG. 1. Immunoprecipitation of extracts from [^{3}H]leucine-labeled HeLa cells. Labeled extracts were first digested with S.a.V8 before immunoprecipitation with serum Ze. Lanes 1 to 7 correspond to extracts digested with 0, 10, 30, 90, 270, 810, or 2,430 μg of protease per ml, respectively. The results showed that two sets of partially protease-resistant polypeptides (X/X′ and Y/Y′) were precipitated by serum Ze. The X/X′ polypeptides were considerably more resistant to protease than were the Y/Y′ polypeptides, which were further degraded to the 20- and 17-kDa polypeptides. There were also less well defined fragments precipitated at the lower M_w range (lanes 5 and 6). KD, Kilodaltons.

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† Publication 4526BCR from the Research Institute of Scripps Clinic.

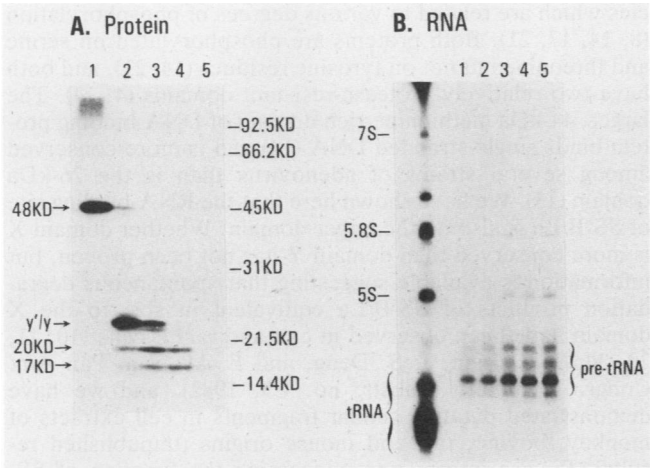

FIG. 2. Immunoprecipitation of protease-digested [^{32}P]phosphate-labeled HeLa cell extract. Labeled cell extracts were first digested with 0, 25, 75, 225, or 675 μg of S.a.V8 protease per ml, corresponding to lanes 1 to 5, respectively, before immunoprecipitation with anti-SS-B/La serum Ze. Half of the immunoprecipitate was treated with RNase and separated by SDS-polyacrylamide gel electrophoresis to reveal the ^{32}P-labeled antigenic polypeptides (A), and the other half was extracted with phenol and then separated on an 8 M urea–10% polyacrylamide gel for the detection of the associated RNAs (B). Left lane in panel B is total phenol-extracted cellular RNA. Panel B is a low exposure, and thus it identifies mainly the precursors of tRNA. SS-B/La-associated precursors of tRNA were coprecipitated independent of the integrity of native 48-kDa or phosphorylated fragments Y/Y'. KD, Kilodaltons.

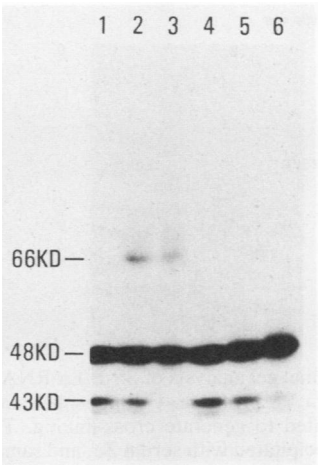

FIG. 3. UV-cross-linked SS-B/La-RNA complexes. Western blotting of cell extracts with serum Ze.. HeLa cells were UV irradiated as described in the text for 0 min (lanes 1 and 4), 3 min (lanes 2 and 5), or 6 min (lanes 3 and 6). Extracts in lanes 4 to 6 were each digested with 4 μg of RNase at 37°C for 30 min. Extracts in lanes 1 to 3 were similarly treated but without the added RNase. The band equivalent to 66 kDa detected in irradiated cell extracts disappeared after RNase digestion. The 43-kDa bands were degradation products of native SS-B/La. KD, Kilodaltons.

binding did not require the intact 48-kDa protein. Detectable Y/Y' and 20- and 17-kDa fragments were also not required for RNA binding because precursors of tRNA were still immunoprecipitated (Fig. 2B, lane 5) when no phosphorylated fragments could be seen (Fig. 2A). At this concentration of S.a.V8, the X fragment (nonphosphorylated) would still be immunoprecipitated by serum Ze (Fig. 1, lane 6). These results directed our attention to the possibility that domain X was involved in RNA binding. The apparent progressive increase in precipitable pre-tRNA is unexplained but is not an artefact due to nonspecific sticking of RNA since controls with normal human serum were negative.

UV cross-linking of in vivo SS-B/La–RNA complex. Cross-linking between SS-B/La and RNA was induced by UV irradiation as described by Dreyfuss et al. (7) with an 8-W lamp (sylvania G8T5) placed 4.5 cm from the cell monolayer for 3 to 24 min to determine the optimum conditions for cross-linking. Stable complexes of SS-B and RNAs were determined by Western blotting (28) after 3 min of irradiation (Fig. 3). An extra band of about 66 kDa was detected from extracts of irradiated cell. This 66-kDa band was not detected when the irradiated cell extract was predigested with 4 μg of RNase before blotting. The signal of this RNase-sensitive band was about 10% that of the 48-kDa band, suggesting that under these conditions, approximately 10% of the cellular SS-B/La protein was cross-linked to RNA.

To determine if the X domain might be cross-linked to RNA, immunoprecipitates from [^{32}P]phosphate-labeled and UV-irradiated HeLa cells were washed twice with 10 mM Tris hydrochloride–1 mM EDTA (pH 7.4) before being mixed with various amounts of protease in 10 μl of 200 mM Tris hydrochloride–2 mM EDTA buffer (pH 6.9) and incubated at 37°C for 30 min. Each sample also contained 2 μg of RNase. Digestion was stopped with the addition of 20 μl of 2× Laemmli sample buffer (15), and the mixture was boiled for 2 min. The normally nonphosphorylated X fragments (Fig. 2A) could be detected as phosphorylated compounds under these conditions (Fig. 4). These experiments do not exclude the possibility that RNA is also complexed to the Y/Y' fragments, since the latter already contain phosphorylated amino acids. However, the data presented in Fig. 2 and the results of diagonal gel electrophoresis described below substantially ruled out this possibility.

Diagonal gel analysis. The method of diagonal polyacrylamide-sodium dodecyl sulfate (SDS) gel electrophoresis has been used to identify monomeric components of cross-linked

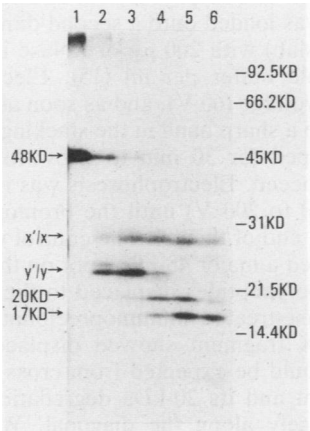

FIG. 4. Immunoprecipitation of protease-digested extracts from [^{32}P]phosphate-labeled and UV-irradiated HeLa cells with the anti-SS-B/La serum Ze. Samples of the immunoprecipitate were digested with 0, 0.15, 0.45, 1.35, 4.05, or 12.15 μg of S.a.V8 protease corresponding to lanes 1 to 6, respectively. RNase (2 μg) was also added to each sample to digest the bulk of covalently bound RNA. The X/X' polypeptides showed the presence of [^{32}P]phosphate label. In the absence of UV irradiation, ^{32}P was not detected on these polypeptides. KD, Kilodaltons.

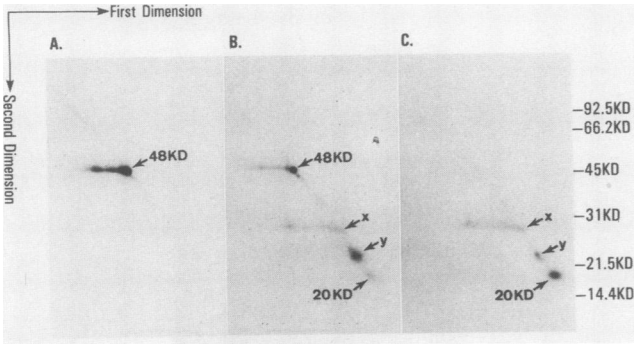

FIG. 5. Diagonal gel analysis of SS-B/La-RNA complex partially digested with S.a.V8 protease. [^{32}P]phosphate-labeled HeLa cells were UV irradiated to generate cross-linking. These cell extracts were immunoprecipitated with serum Ze, and samples were digested with 0, 0.45, or 1.35 μg of protease (A, B, and C, respectively). Samples were first separated by an SDS–15% polyacrylamide gel electrophoresis system. This gel was stained and destained rapidly; gel strips were cut out, and each was loaded onto a second SDS–20% polyacrylamide gel electrophoresis system with the addition of RNase in the sample buffer. Polypeptides migrating off the diagonal represent RNA-binding fragments. The results showed that RNAs were bound to the X polypeptides but not to the Y polypeptides or the degradation products of Y. KD, Kilodaltons.

complexes after cleavage of the cross-linking bond (12). The procedure is a two-dimensional electrophoresis separation utilizing the size dependence of protein mobility in SDS to distinguish cross-linked from non-cross-linked proteins. In such a system, non-cross-linked proteins fall on a diagonal line and cross-linked proteins are displayed away from the diagonal. In this experiment, RNase was used as the cleavage reagent to reduce UV-induced protein-RNA complexes to monomeric polypeptides. ^{32}P-labeled HeLa cells were UV irradiated, and immunoprecipitates were obtained in the usual manner. Equal samples of the immunoprecipitates were digested with 0, 0.45, and 1.35 μg of S.a.V8 protease and electrophoresed in the first dimension on a 1-mm-thick 15% gel slab in a typical SDS-polyacrylamide gel electrophoresis system (15). Gel strips were cut and adjusted to pH 6.9 (6), and each was loaded onto a second dimension (1.5-mm-thick 20% gel slab) with 200 μg of RNase in 0.1% SDS–1× Laemmli sample buffer per ml (15). Electrophoresis was started at low voltage (60 V), and as soon as the sample was concentrated to a sharp band in the stacking gel, electrophoresis was stopped for 30 min to allow for in situ RNase digestion to proceed. Electrophoresis was restarted at regular voltage (100 to 200 V) until the bromophenol blue dye migrated to the end of the gel. The control without protease (Fig. 5A) showed a major 48-kDa spot on the diagonal, with other 48-kDa polypeptides displaced to the left of the diagonal. In protease-treated immunoprecipitates (Fig. 5B and C), only the X fragment showed displacement from the diagonal, as would be expected from cross-linked proteins. The Y fragment and its 20-kDa degradation product were localized precisely along the diagonal. We repeated this experiment with [^{35}S]methionine label instead of [^{32}P]phosphate and showed that the signal migrating off the diagonal also contained methionine. These results confirmed that RNA was bound to domain X and not to domain Y.

We have previously called attention to the structural similarities between SS-B/La and the 72-kDa DNA-binding protein of adenovirus (4). Both DNA-binding protein and SS-B/La are phosphoproteins with several isoelectric species which are related to various degrees of phosphorylation (8, 14, 17, 21). Both proteins are phosphorylated on serine and threonine but not on tyrosine residues (14, 21), and both have two relatively protease-resistant domains (4, 14). The larger, 44-kDa methionine-rich domain of DNA-binding protein binds single-stranded DNA (14) and is more conserved among several strains of adenovirus than is the 26-kDa domain (13). We have shown here that the RNA-binding site of SS-B/La is also on the larger domain. Whether domain X is more conserved than domain Y has not been proven, but information is available suggesting that spontaneous degradation products of SS-B/La equivalent in size to the X domain have been observed in calf and rabbit cells (10, 11, 20, 30; T.-S. Lieu, J. S. Deng, and E. M. Tan, Pan Am. Congr. Rheumatol., abstr. no. C9, 1982), and we have demonstrated putative similar fragments in cell extracts of monkey, bovine, rat, and mouse origins (unpublished results). Precise information concerning the function of SS-B/La is lacking, though there is some evidence to show that it may be involved in transport and processing of small nuclear and cytoplasmic RNAs (18, 24). The cloning of the SS-B/La gene may help to shed light on the function of the protein (2).

Diagonal gel electrophoresis has been used for the identification of disulfide-bond-cross-linked proteins in complexes such as ribosomes (12). We have adapted this system for the study of the interaction between RNA and protein. This technique may be of general use to examine RNA-binding proteins that can be UV cross-linked to their RNA species. We are currently using this technique to examine the RNA-binding proteins in other small nuclear ribonucleoprotein complexes.

E.K.L.C. is a recipient of an Arthritis Foundation Postdoctoral Fellowship. This work is supported by an Alberta Heritage Foundation for Medical Research Fellowship Award and by Public Health Service grants AM32063 and AI10386 from the National Institutes of Health.

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