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Detection of SS-A/Ro and SS-B/La Autoantibodies Using Immunoblotting Procedure and Characterization of the Antigens from 293 Cells and KB Cells

Yasunori INAGAKI, Youichirou HAMASAKI, Yoko JINNO, Keiichi HOSOKAWA* and Hiroaki UEKI

*Department of Dermatology and *Department of Biochemistry, Kawasaki Medical School, Kurashiki 701-01, Japan*

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ABSTRACT. Specific and sensitive assay was performed to detect both antiSS-A/Ro and antiSS-B/La antibodies in sera of patients with collagen diseases including SLE, PSS, etc. The SS-A/Ro and SS-B/La antigens were prepared from human spleen (HSE) and cultured human cell line (KB cells), while rabbit thymus extract (RTE) was used as SS-B/La antigen marker.

The antigens were partially purified by DEAE cellulose column chromatography. The SS-A/Ro antibody was shown to react mainly with 58KDa peptide by means of immunoblotting. Sera containing both the SS-A/Ro and SS-B/La antibody reacted with 40KDa peptide of RTE, and 58KDa, 42KDa and 40KDa peptides of HSE. We found that some of SS-A/Ro antisera could further react with 64KDa peptide in HSE. The 58KDa peptide is rich in a cytoplasmic fraction of KB cells, and the 40KDa peptide in the nucleoplasmic fraction. KB cells are not less good source of the antigens than human spleen.

Extracts of 293 cells (human embryonic kidney cells expressing adenovirus-5 E1 gene) were prepared by the same method from KB cells, though immunoblotting patterns of both SS-A/Ro and SS-B/La antigens of 293 cell extracts are similar to those of KB cells, the relative content of SS-B/La antigens in 293 cell extracts are decreased.

Key words : SS-A/Ro antibody — SS-B/La antibody — autoimmune diseases — adenovirus E1 gene — 293 cells

Autoantibodies frequently appear in sera of patients with systemic lupus erythematosus (SLE), progressive systemic sclerosis (PSS), Sjögren's syndrome (SS) and other connective tissue diseases. Although there are many varieties of autoantibodies, their role of pathogenicity is still unclear.

SS-A/Ro and SS-B/La antibodies are important autoantibodies in relation to sicca syndrome and photosensitivity for SS-A/Ro antibody,[1] and viral infection for the SS-B/La antibody.[2] To investigate these antibodies and the corresponding antigens in molecular detail a sensitive and specific method is needed. In addition the procedure will be useful to make the precise diagnosis.

Partial purification of SS-A/Ro and SS-B/La antigens from human spleen and a highly sensitive detection system with using immunoblotting method was reported by Herrera-Esparza *et al.*[1,3] in 1986. The antigens detected by the

稲垣安紀，浜崎洋一郎，神野陽子，細川桂一，植木宏明

specific system are probably similar to those of Deng *et al.*[4] We believe that this method is sensitive enough and reliable to detect the SS-A/Ro and/or SS-B/La antibodies. Furthermore, additional polypeptide of 64KDa that reacted with some of antiSS-A/Ro positive sera was detected.

However, human spleen, as a source of the antigens, is not always preserved. The antigen preparation was performed from cell lines instead of human spleen.

The SS-B/La antigen reacts with adenovirus virus-associated RNA (VA RNA)[2] and Epstein-Barr virus RNA.[5] Investigations of them were already performed using Epstein-Barr virus transformed cell lines and adenovirus infected cells, but not with adenovirus transformed cell line. We confirmed the presence of both SS-A/Ro and SS-B/La antigens in extracts of 293 cells which was transformed by adenovirus E1 oncogenes.[6]

MATERIALS AND METHODS

Sera

Sera used in this study were obtained from patients with systemic lupus erythematosus (SLE), progressive systemic sclerosis (PSS) and Sjögren's syndrome (SS). These sera have been shown to have activity towards SS-A/Ro and/or SS-B/La antigen(s). Reference sera with apparently monospecific antibodies to the antigens SS-A/Ro and SS-B/La were provided by the Center for Disease Control (Atlanta, GA, USA). Normal controls were also included. Patient's sera were examined for antibody activity against human spleen extract and rabbit thymus extract, comparing to the result of reference sera on double immunodiffusion (DI) and counterimmunoelectrophoresis (CIE). The patient's sera gave precipitin lines of identity only with antiSS-A/Ro and/or antiSS-B/La antibodies.

Cells

KB cells; human laryngeal carcinoma cell line, and 293 cells; human embryonal kidney cells transformed by transfection with human adenovirus DNA,[6] were used in this study.

They were grown respectively in monolayers, using Eagle minimal essential medium (Flow Laboratories, Inc., Rockville, Md.) supplemented with 7% fetal calf serum (FCS: Flow Laboratories, Inc., Rockville, Md.).

Both cells were harvested by washing monolayer cells with 10 mM phosphate buffered saline (pH 7.2) (PBS) containing 0.02% ethylenediaminetetraacetic acid (EDTA) to avoid any proteolytic effects, followed by centrifugation at 1,000 $\times$g for 15 min at 4°C.

Preparation of extracts

Acetone extract of rabbit thymus (Pel-Freeze Biologicals, Rogers, Arkansas) was used as the source of SS-B/La antigen.[7] For 60 mg of rabbit thymus acetone powder, 1 ml of 10 mM PBS (pH 7.2) with 0.5 mM phenylmethylsulfonyl fluoride (PMSF; Sigma Chemical Co., St. Louis, Mo.) was added. Antigens were extracted by gently stirring at 4°C for 6 hr followed by centrifugation at 10,000 $\times$g for 10 min at 4°C. The supernatant was used as rabbit thymus extract (RTE).

Human spleen extract (HSE) was prepared from normal human spleen following the procedure described by Clark *et al.*[8] and Herrera-Esparza *et al.*[3]

with our minor modifications. Minced spleen specimens were homogenized in 150 mM NaCl, 50 mM Tris-HCl (pH 7.2) and 1.0 mM PMSF with a tight fitted glass-teflon homogenizer for 3 min on ice. Fibrous material was removed from the homogenate and homogenized in the same buffer twice with the Polytron tissue homogenizer (Kinematica, Switzerland) for 3 min at the strength of range 6 on ice. The supernatant, after clarification by centrifugation at 12,000 ×g at 4°C for 40 min, was collected as the source of HSE for further purification.

Cell extracts were prepared from two human cell lines, KB and 293 cells. Fractionation of the cells were performed in such a manner as follows. Cells were harvested, and washed with 10 mM PBS (pH 7.2), 0.5 mM PMSF by centrifugation at 1,000 ×g for 5 min at 4°C. Pelleted cells were resuspended in 20 times packed cell volume of reticulocyte standard buffer (RSB: 10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl) (pH 7.4)[9] containing 0.1 mM PMSF, left on ice for 10 min, followed by centrifugation at 600 ×g for 5 min at 4°C. The supernatant was collected as cytoplasmic fraction (KBE1). Pelleted nuclei were resuspended in the same volume of RSB with 0.5 mM PMSF, 0.3% Nonidet P-40 (Sigma Chemical Co., St. Louis, Mo.), and 0.2% deoxycholic acid sodium salt (DOC: Sigma Chemical Co., St. Louis, Mo.) followed by homogenization with 20 strokes of a tight fitted (0.04 mm clearance) teflon homogenizer. The homogenate was centrifuged at 1,200 ×g for 5 min at 4°C. The supernatant was designated as treated a nucleoplasmic extract (KBE2).

Partial purification of extracts

Extracts were partially purified by DEAE (DE52: Whatman Paper Ltd., England) column chromatography. The crude extracts were centrifuged at 12,000 ×g for 30 min at 4°C and the supernatant was dialyzed against autoclaved solution of 150 mM NaCl, and 50 mM Tris-HCl (pH 7.2) overnight at 4°C using a dialysis membrane tubing which cutoffs molecules smaller than 12KDa. The samples were applied to a DEAE-cellulose column equilibrated with 150 mM NaCl, 50 mM Tris-HCl (pH 7.2). Fractions of 2.5 ml were collected by micro fractionator (Gilson Co., model FC-80K). Protein concentration of the eluants were monitored by a single path monitor (Pharmacia Co., model UV-1) with absorbance at 280 nm. The polypeptides were eluted by stepwise increase in the concentration of NaCl up to 0.5 M in the same buffer. The SS-A/Ro and SS-B/La antigens were identified by double immunodiffusion[10] (*e.g.* Ouchterlony,[11] 1958) and counterimmunoelectrophoresis[7] at each step of the purification. Fractions with SS-A/Ro and SS-B/La antigenicity were collected with immediate addition of PMSF to a final concentration of 0.5 mM. Aliquot was taken from each fraction, dialyzed against autoclaved double distilled water for 12 hr at 4°C and lyophilized. The antigens were stored at −70°C.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Extracts were subjected to electrophoresis in 8% or 10% SDS polyacrylamide gel slab (1 mm thickness, 10×16 cm) (*e.g.* Laemmli, 1970[12]) in duplicate. Samples were heated in SDS-sample buffer containing 2% SDS and 5 mM β-mercaptoethanol at 100°C for 2 min. After a 4 hr-run at 20 mA, polypeptide bands were stained with coomassie brilliant blue R (Sigma Chemical Co., St. Louis, Mo.) on one gel slab, and the other gel served for electroblotting.

Immunological detection of SS-A/Ro and SS-B/La antigens

Double immunodiffusion was performed using 0.6% agarose gel prepared in 25 mM barbiturate buffer (pH 8.6) at each step of purification. Counter-immunoelectrophoresis[7] was carried out for sensitive detection of anti SS-A/Ro and SS-B/La antibodies with minor modifications. Agarose was prepared in barbiturate buffer (pH 8.4) of 0.1μU (ionic strength). Electrophoresis was performed in barbiturate buffer (pH 8.4) of 0.05μU (ionic strength), with a current of 4 mA per slide. The polypeptide preparations were examined by immunoblotting procedure to dissolve into antigenic bands on slab gel according to Towbin's procedure (*e.g.* Towbin, 1977). The SDS-polyacrylamide gel was soaked in blotting buffer, 192 mM glycine, 20% methanol. 25 mM Tris (pH 8.3) for more than 1 hr at 4°C to remove SDS, followed by electrotransfer of the polypeptides to a nitrocellulose membrane. Electrotransfer was carried out at 25°C and at a constant voltage of 60 V 5 hr in blotting buffer using a Bio-Rad trans-blot cell. The blotted nitrocellulose membrane was incubated in TPBS, 10 mM PBS with 0.05% Tween 20 (Sigma Chemical Co., St. Louis, Mo.) for 1 hr at room temperature and overnight at 4°C to saturate additional protein binding sites of the membrane. Diluted (1:100) sera containing SS-A/Ro and/or SS-B/La antibodies were allowed to react with antigens on the membrane at 25°C for 1 hr. After extensive washing with TPBS, human IgG fixed on the membrane were detected by avidin-biotin-peroxidase complex (ABC) method (Vector Lab. Inc., Burlingame, CA).

RESULTS

SS-A/Ro and/or SS-B/La antigens were successfully prepared from human spleen, rabbit thymus acetone powder, KB cells and 293 cells. The procedure for extraction was highly reproducible and reliable. Presence of PMSF (inhabitor of serine type proteases) well prevented proteolysis during the extraction. The bands on the immunoblotted membrane, which reacted with the standard sera, did not change at anytime of extraction, as reported by Herrera-Esparza *et al.*[1] Analysis by SDS-PAGE was shown in Fig. 1.

By DEAE cellulose column chromatography, both SS-A/Ro and SS-B/La antigens were eluted by 300 mM NaCl, 50 mM Tris HCl (pH 7.2). These fractions were used as partially purified antigens.

Counterimmunoelectrophoresis was used to detect the antigen from cell extracts at each step of extraction because of high sensitivity compared to double immunodiffusion method (Fig. 2).

Immunoblotting patterns of several antigens were shown in Fig. 3. Antisera containing both SS-A/Ro and SS-B/La antibodies reacted with polypeptides of 58KDa, 42KDa and 40KDa. They are in common with HSE, KBE2, and 293 cell extract (293E). RTE showed up a clear band of 40KDa peptide which is assigned as SS-B/La antigen. SS-A/Ro reference sera did not react with any peptides of RTE (Fig. 4, panel 1-A), but with 58KDa polypeptides of HSE (Fig. 4, panel 1-B), KBE1, KBE2 and 293E (not shown). Some of the antiSS-A/Ro antisera could additionally react with 64KDa peptide in HSE and KBE (Fig. 4). The cytoplasmic fraction of KB cells (KBE1 in Fig. 3) strongly reacted with 58KDa polypeptides, while nucleoplasmic extract of KB cells (KBE2) did

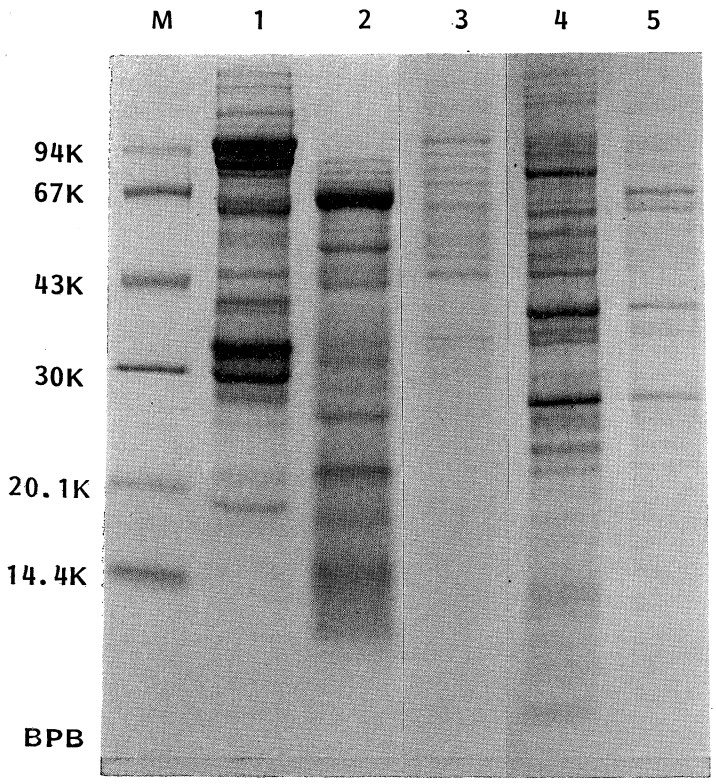

Fig. 1. Coomassie brilliant blue-stained polypeptides of extracts from human spleen (lane 1), rabbit thymus acetone powder (lane 2), 293 cells (lane 3), cytoplasmic fractions of KB cells (lane 4) and nucleoplasmic fractions of KB cells (lane 5) separated by 8% SDS-PAGE. Molecular weight marker were shown in lane M (94K: phosphorylase b, 67K: bovine serum albumin, 43K: ovalbumin, 30K: carbonic anhydrase, 20.1K: soybean trypsin inhibitor, 14.4K: α-lactalbumin)

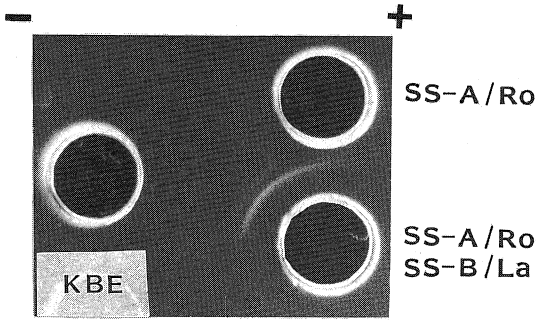

Fig. 2. Counterimmunoelectrophoresis of KB cells extract (KBE). The two precipitin lines obtained with SS-A/Ro and/or SS-B/La reference sera.

with 58KDa and 40KDa bands. The preparation of 293E was the mixture of cytoplasmic and nucleoplasmic fractions of 293 cells to get enough antigenic polypeptides. The distributions of SS-A/Ro and SS-B/La antigens were similar to those of KB cells. As shown in Fig. 3, 58KDa antigen was clearly recognized on immunoblotting of 293E. In 293E, this 58KDa polypeptide seems to be the main antigen in contrast to HSE and KBE2. The 42KDa and 40KDa bands were weakly identified by this method.

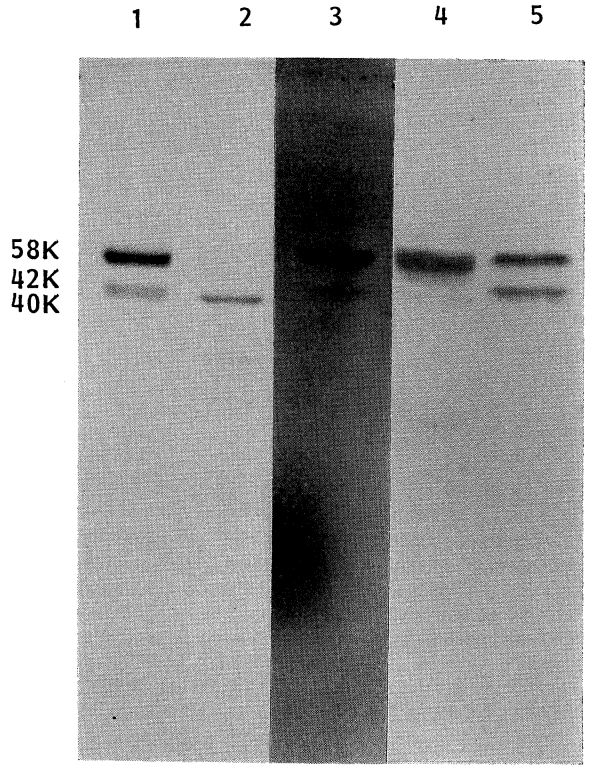

Fig. 3. Immunoblotting analysis of the SS-A/Ro and SS-B/La antigens. Lane 1: partially purified human spleen extract, Lane 2: partially purified rabbit thymus extract, Lane 3: extracts from 293 cells. Lane 4: cytoplasmic fraction of KB cells and Lane 5: nucleoplasmic fraction of KB cells. The SS-A/Ro antigens (58KDa) and SS-B/La antigens (42KDa, 40KDa) were detected by reference serum containing both SS-A/Ro and SS-B/La autoantibodies.

Comparative study of the double immunodiffusion and immunoblotting were performed (Fig. 4). Titration on double immunodiffusion of the sera were carried out and described below the panels in Fig. 4.

The panel 1 on Fig. 4 showed the results of immunoblotting using SS-A/Ro monospecific antibody. This revealed the presence of two bands, 64KDa and 58KDa, only in KBE. Other monospecific antiSS-Ro antibodies were divided into 2 groups. One group recognized only 58KDa peptide in KBE, others recognized both 64KDa and 58KDa peptides in KBE. All of them did not

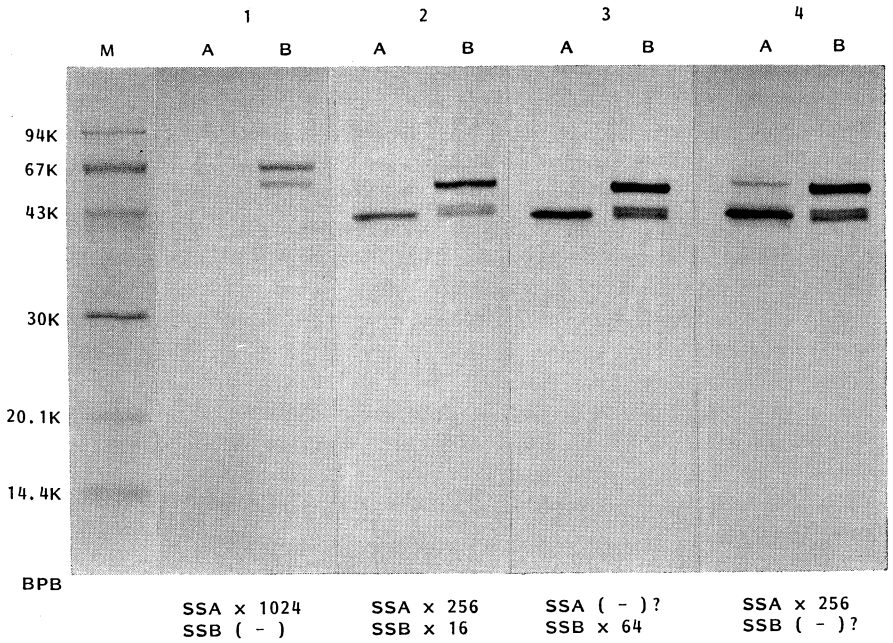

Fig. 4. Comparison of immunoblotting analysis and double immunodiffusion. Molecular weight markers were the same as those in Fig. 1 (lane M). Panels 1 and 2 were reacted with SS-A/Ro or SS-A/Ro and SS-B/La reference antibodies. Each panel was analyzed using sera of suspected patients (panels 3, 4). The results of double immunodiffusion method were listed below the panels. A; RTE, B; KBE

reveal the presence of any bands in RTE. In panel 2 in Fig. 4 there are bands reactive with both SS-A/Ro and SS-B/La positive antisera. The 64KDa band was not observed for KBE. The serum use in panel 3 (Fig. 4) resembled only SS-B/La antigenicity by double immunodiffusion (DI) and counterimmunoelectrophoresis (CIE), but the peptides developed by immunoblotting resembled SS-A/Ro antigenicity, and the patterns were similar to those of panel 2 (Fig. 4). Serum of the panel 4 (Fig. 4) did not show any activity of SS-B/La antibody on DI and CIE, but it was similar to the pattern of panel 2 (Fig. 4), and additional 59KDa band of RTE was observed. These four immunoblotted panels were processed at the same time and under the same conditions.

DISCUSSION

The SS-A/Ro and/or SS-B/La antigens were successfully prepared from human spleen, rabbit thymus acetone powder, KB cells and 293 cells. The immunoblotting method is satisfactorily sensitive to obtain SS-A/Ro and SS-B/La antibodies from patient's sera (Fig. 4). The results are similar to those of Herrera-Esparza *et al.*[1,3] and Deng *et al.*,[4] in which antiSS-A/Ro antibody reacted with 58KDa polypeptide and antiSS-B/La antibody with both 42KDa and 40KDa bands by the immunoblotting using HSE. We believe that these corresponded to the approximate molecular weight of the antigens reported by the above scientists.[1,3,4] The immunoblotting procedure and partial purification

of HSE for the identification of SS-A/Ro and SS-B/La antibodies were initially reported by Herrera-Esparza *et al.* and we could confirm their results. Moreover, we found some of the antiSS-A/Ro antisera could additionally react with 64KDa polypeptide in HSE and KBE. This will indicate a heterogeneity of the autoantibodies or presence of unknown autoantibodies. Recently, two distinct subsets of lupus erythematosus (LE): Subacute cutaneous LE and neonatal LE were presented in dermatological field, describing their high association with SS-A/Ro antibody.[13] Therefore, highly sensitive and easily reproducible method to detect the antibodies was needed. Also, the monospecific antisera will be necessary in future so that the method may be much more sensitive. To propagate this method, the establishment of a method to prepare antigen will become important. Though the human spleen is a good source of antigens, it is not always preserved. KB cells were not less a good source than the human spleen, since the cell line is the same human origin. In the cells, SS-A/Ro antigen was rich in the cytoplasmic fraction, while SS-B/La antigens were rich in its nucleoplasmic fractions.

The 293 cells were selected to examine the relationship between cellular and viral antigens. The 293 cells are transformed human embryonic kidney cells with oncogenes of adenovirus 5 inserted and being expressed permanently.[6] Some authors suggest the relationship of the antigens (especially SS-B/La antigens) with viral RNA, encoded by Adenovirus[14] or Epstein-Barr (EB) virus genome.[5] The virus related SS-B/La antigens were prepared from WiL2 cells[4] and Raji cells,[5] which were transformed by EB virus, and from human adenovirus infected KB cells[15] or HeLa cells.[16] The KB cells and HeLa cells in the previous reports are permissive hosts for adenovirus, and allows a productive infection. We should further investigate SS-B/La antigens in cells which transformed by adenovirus using 293 cells. The SS-B/La antigen binds specifically to virus associated RNA (VA RNA)[14] transcribed by RNA polymerase III.[17] The E1 gene is an oncogene consisting of two elements, E1A and E1B which are encoded by adenovirus genome.[17] The 293 cells do not contain adenovirus genes encoding VA RNA and others except for E1A and E1B region. E1A codes for phosphorylated polypeptides 53KDa, 44KDa and so on.[18] Their molecular weights are similar to those of SS-A/Ro and SS-B/La antigens. Chan *et al.*[19] speculated that SS-B/La antigen showed striking similarity to the adenovirus 72KDa DNA-binding protein which is assigned by adenovirus E2A genome.[18] The E2A gene is expressed early in infection as E1 gene. We therefore speculated that the amounts of SS-B/La and/or SS-A/Ro antigens might be increased in 293 cells, but contrary to our expectation they were not increased as demonstrated in our preliminary experiments. However, there are many interesting problems to investigate the relationship between viral infection and transformation, and autoimmune diseases. This system will become useful to solve the problem in future.

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