DESCRIPTION AND PARTIAL CHARACTERIZATION OF A NUCLEOLAR RNA–ASSOCIATED AUTOANTIGEN DEFINED BY A HUMAN MONOCLONAL ANTIBODY

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A valuable approach to the characterization of known and novel autoantigens has used cell component–reactive monoclonal antibodies (mAb) derived from immortalized, in vivo–primed, autoreactive B lymphocytes. For example, in animal systems, murine mAb have been produced from spleen cells of autoimmune inbred mouse strains and used to characterize the autoantigenic structures of Sm (1, 2) and nRNP (3, 4), as well as DNA (5–12) and RNA (13). Similarly, human mAb derived from patients with autoimmune disorders have provided valuable insights into the autoantigenic determinants of the DNA molecule, demonstrating unique and crossreactive features (14–17). However, although there have been human mAb generated that are reactive with non-DNA autoantigenic moieties (15, 18), progress in this area has been considerably slower.

This report describes an autoantibody-producing B cell clone, immortalized from a patient with systemic lupus erythematosus (SLE), that secretes an IgM mAb reactive with DNA and a salt-extractable autoantigen found primarily in the nucleolus. This antigen is no longer bound by the mAb after in situ treatment of the cells with either RNase or trypsin, binds avidly to DEAE-cellulose, and appears to consist of at least two polypeptide chains, of 17 and 18 kD. The following studies suggest that the antigen probably is not related to conventional RNA-associated autoantigens and may represent a novel system. A portion of these studies has been presented previously (19).

Materials and Methods

Isolation and Activation of Donor Peripheral Blood Mononuclear Cells (PBMC)

Sterile, heparinized venous blood was obtained from a patient (DW) with active, untreated systemic lupus erythematosus (SLE) followed at The Rockefeller University Hospital. This blood was separated into PBMC by centrifugation on a Ficoll-Paque cushion (ρ 1.077; Pharmacia Fine Chemicals, Piscataway, NJ). PBMC were divided further into T and non-T cell fractions by the neuraminidase-treated sheep erythrocyte rosetting technique (20). Non-T cells were cultured at a concentration of 2×10^6 cells/ml for 3.5 d in the presence of fixed *Staphylococcus aureus*, Cowan strain I (0.01% final concentration; Calbiochem-Behring Co., San Diego, CA). Before hybridization, viable cells were isolated by another Ficoll-Paque gradient centrifugation step.

Cell Line Cells

Lymphoid cells were cultured in RPMI 1640 medium supplemented with 10% FCS (Reheis Chemical Co., Phoenix, AZ), glutamine (4 mM), penicillin and streptomycin (100 U/ml), and kanamycin (50 mg/ml). The hypoxanthine/guanine phosphoribosyl transferase (HGPRT)[1]-deficient, EBV-transformed, human B lymphoblastoid cell line, 0467.3, was prepared by subcloning GM 0467 (Genetic Mutant Cell Repository, Camden, NJ) as previously described (21). These cells were cultured at 10^5 cells/ml in culture medium containing 8-azaguanine for 5 d before hybridization. The human laryngeal epidermoid carcinoma cell line, HEp-2 (22), was obtained from the Genetic Mutant Cell Repository and cultured as recommended.

Generation of Human mAb

Human × human B cell hybridomas were constructed by somatic cell hybridization techniques as previously described (21). Briefly, mitogen-activated human B lymphoblasts were fused with the HGPRT-deficient human B cell variant, 0467.3, at a 1:1 ratio, by exposure for 2 min at room temperature to 0.5 ml of 40% PEG (~1,000 mol wt; J. T. Baker Chemical Co., Phillipsburg, NJ) followed by centrifugation for 4 min at 1,000 rpm. After two washes, cells were dispensed into 96-well plates at a density of 5×10^4 parental tumor cells per microwell in culture medium containing hypoxanthine (10^{-4} M), thymidine (5×10^{-5} M), and aminopterin (10^{-7} M) (HAT). Cultures were fed with the same medium approximately every third day for 3 wk and then with medium without aminopterin until assaying for Ig content and antigen-binding activity.

Enzyme-linked Immunosorbent Assays (ELISA)

Anti-DNA ELISA. Antibodies to DNA were detected as previously reported (23). Briefly, 96-well polystyrene plates (Costar, Cambridge, MA) were exposed to 0.001% salmon protamine sulfate (Sigma Chemical Co., St. Louis, MO) for 1 h at room temperature, washed with distilled water, and then reacted for 16 h at 4°C with 150 μl of calf thymus DNA (Worthington Biochemical Corp., Freehold, NJ) at 5 μg/ml in PBS. At this point, plates were washed, blocked with culture medium for 1 h, and then incubated with mAb or human sera with known levels of reactivity to DNA for 18 h at 4°C. After washing three times, affinity-purified, peroxidase-labeled, goat anti–human $\kappa + \lambda$ antibodies (Tago, Inc., Burlingame, CA) were added for 1 h at 37°C. The assay was developed for 1 h at 37°C with 4-aminoantipyrene/phenol substrate (24).

Anti–Extractable Nuclear Antigen (ENA) ELISA. Antibodies to an assortment of salt-extractable antigens from rabbit thymus were detected as previously described (25). The crude soluble antigenic extract was obtained by 0.15 M NaCl extraction of rabbit thymus acetone powder (RTE) (Pel Freez Biologicals, Rogers, AR) for 3 h at 4°C; in certain experiments, this crude extract was fractionated by NaCl gradient (0.05–0.5 mM) elution from DEAE-cellulose column (DE52; Whatman, Inc., Clifton, NJ) as described (25).

Fluorescent Antinuclear Antibody (ANA) Assay

Antibodies to cellular antigens were detected by a double sandwich immunofluorescent antibody technique using human HEp-2 cells as the substrate. After 48 h of growth on coverslips, HEp-2 cells were treated with cold 100% methanol for 5 min at −20°C, and then with 0.5% Triton X-100 in PBS for 1 min at room temperature. The fixed, permeabilized cells were incubated with fresh culture medium for 2 h at room temperature, washed, and reacted for 18 h with cell culture supernatants containing various human monoclonal antibodies or with serum containing reference autoantibodies. After three washes with fresh medium, cells were exposed to 40 μg/ml of affinity-purified rabbit anti–human Ig antibodies for 1 h at room temperature. Subsequently, cells were washed three times and then reacted with an FITC-labeled goat anti–rabbit antibody preparation

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[1] *Abbreviations used in this paper:* ANA, antinuclear antibody; DRB, 5,6-dichloro-1-β-ribofuran-osylbenzimidazole; dsDNA, double-stranded DNA; ENA, extractable nuclear antigen; HGPRT, hypoxanthine/guanine phosphoribosyl transferase; RTE, rabbit thymus extract; ssDNA, single-stranded DNA.

that had been preabsorbed to remove reactivity with cellular constituents (the gift of Dr. E. Wang of The Rockefeller University).

Antibodies reactive with native DNA were detected by indirect immunofluorescence using fixed *Crithidia luciliae* organisms (AnDNA FluoroKit II; Clinical Sciences Inc., Whippany, NJ) according to the directions of the manufacturer.

Rabbit antibodies reactive with mammalian RNA polymerase I were kindly provided by Dr. Kathleen Rose of The University of Texas Health Science Center at Houston. Indirect immunofluorescent assays were performed with these antibodies and a rhoda-mine-conjugated preparation of goat antibodies to rabbit Ig (Tago, Inc.).

Treatment of HEp-2 Cells with 5,6-Dichloro-1-β-ribofuranosylbenzimidazole (DRB), Actinomycin D, DNase or RNase, and Trypsin

DRB and actinomycin D were purchased from Calbiochem-Behring Co. For studies using these compounds, HEp-2 cells were cultured for 48 h at 37°C in 5% CO_2 atmosphere and then incubated with either DRB, at concentrations of 25, 5, or 1 µg/ml for 6 h, or with actinomycin D, at concentrations of 5.0, 0.5, and 0.05 µg/ml for 2 h. Under the conditions described, the addition of these compounds did not grossly alter the growth characteristics or cell viability of the HEp-2 cells. At the end of the incubation periods, cells were washed, fixed, and then used for the ANA assay.

Deoxyribonuclease I (DNase) and pancreatic ribonuclease A (RNase) were purchased from Sigma Chemical Co. DNase contaminating the RNase preparation was inactivated by heating as described (26). In certain experiments, HEp-2 cells were treated for 30 min at 22°C with 50 µl of 50 µg/ml of either DNase or RNase in phosphate-buffered saline before staining with various antibody preparations (27).

Immunoprecipitation and SDS-PAGE Analysis of ^{35}S-labeled Cellular Antigens

The human myeloid cell line, K562, was cultured for 16 h at 37°C at a concentration of 4 × 10⁶ cells/ml in methionine-deficient RPMI 1640 medium containing 50 µCi of [^{35}S]methionine (~1,100 Ci/mmol; New England Nuclear, Boston, MA), and 10% dialyzed FCS. A cell extract was prepared and immunoprecipitations were performed with human autoimmune sera as described (28). A modification of this procedure was used for immunoprecipitations with human mAb-containing supernatants. For immunoprecipita-tions, 5–10 ml of culture supernatant containing the appropriate mAb or 10 µl of patient serum was rotated with the extract of 10⁶ cells for 14 h at 4°C. The immune complexes were reacted for 2 h at 4°C with protein A–Sepharose beads (Pharmacia Fine Chemicals) that had been coated for 16 h with affinity-purified rabbit anti–human Ig antibodies. The immunoprecipitates were washed and proteins were eluted by boiling for 5 min in 0.25 M sucrose, 2.5% SDS, 0.1 M Tris, pH 7.5, 5.0 mM EDTA, 0.1 M dithiothreitol, 0.005% bromphenol blue, followed by analysis on 12.5 or 15% SDS-polyacrylamide gels. The gels were stained with Coomassie blue, destained, and fluorographed as previously described (29).

Results

Patient Description.   The cell lines described in this report were derived using the peripheral blood B lymphocytes of a 15-yr-old black woman followed at The Rockefeller University Hospital for SLE. The patient had a 3-yr history of intermittent high fevers, joint pains and swelling, oral ulcers, and occasional mild proteinuria. Pertinent laboratory data included erythrocyte sedimentation rate 32 mm/h, hemoglobin, 9.1 gm%, white blood cell count, 2,800 with a normal differential count, total serum protein, 8.6 g/dl, with an elevated serum IgG, 2,600 mg% (nl < 1,800).

The patient's serum contained ANA at a dilution of 1:1,280, with bright, homogeneous immunofluorescent staining of the nucleoli, granular staining of
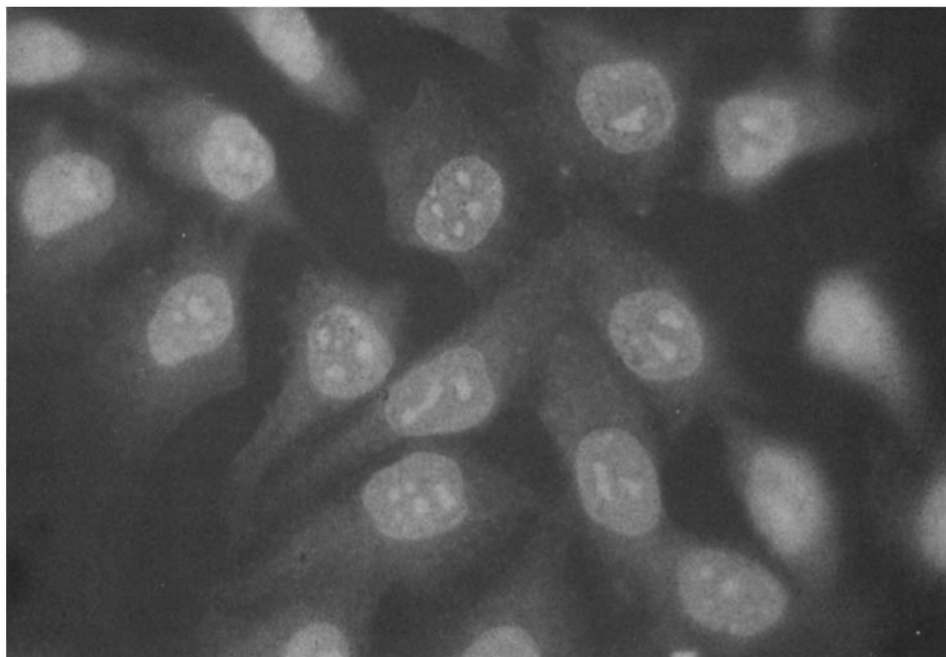
FIGURE 1.   Indirect immunofluorescent staining of fixed HEp-2 cells using the serum (diluted 1:20) of patient DW. Original magnification × 400.

the nucleoplasm, and weak cytoplasmic fluorescence (Fig. 1). Solid-phase enzyme immunoassay and fluid-phase Farr radioimmunoassay revealed significant serum antibody binding to heat-denatured (1:30,000 and 1:1,000, respectively) and native (1:6,000 and 1:250, respectively) DNA. Antibodies to native DNA were confirmed in a *Crithidia luciliae* immunofluorescent assay that showed characteristic staining of kinetoplasts at a serum dilution of 1:640. In addition, antibodies to the battery of ENA contained in RTE were detected by ELISA (1:2,500); these antibodies reacted in hemagglutination assay (30) with antigenic sites that were sensitive to RNase digestion, which were found subsequently to be distinct from the nRNP determinants. Finally, serum autoantibodies reactive with Ro/SSA and Ku (p70/p80) antigens also were detected by immunoprecipitation of radiolabeled cellular RNA or proteins, respectively.

Generation of Human B Cell Lines Reactive with Autoantigens.   B lymphocytes isolated from the peripheral blood of this patient were activated for 4 d by 0.01% formalinized *Staphylococcus aureus*, Cowan strain 1 (protein A–positive) organisms and then fused, in the presence of polyethylene glycol, with the HGPRT-deficient variant human B cell line 0467.3. After 3 wk of selection in HAT medium, 97 growth-positive wells were obtained and tested for production of human Ig and for reactivity with various autoantigens.

Table I lists a series of monoclonal antibodies secreted by certain of these 97 hybridomas and the parental cell line (0467.3) and their reactivities with single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), and a crude ENA preparation. 36 of the 97 growth-positive cultures produced human IgM mAb

TABLE I

Reactivities of Human Monoclonal Antibodies with DNA and Rabbit
Thymus Extract

Clone	Absorbance at 492 nm in ELISA		
	ssDNA	dsDNA	RTE
W8	0.40	0.32	1.64
W99	0.32	0.07	0.07
W4	0.47	0.25	0.0
W20	1.29	0.86	0.0
W48	0.82	0.61	0.0
W64	0.56	0.44	0.0
W15	0.19	0.13	0.0
W76	0.09	0.09	0.0
0467.1	0.05	0.09	0.0
Culture medium	0.0	0.0	0.0

reactive with either native and/or ssDNA, although reactivity with the latter was usually more intense. Initially, two cultures, W8 and W99, were found also to react in ELISA with ENA (Table I). However, after several more weeks of culture, only the W8 cell line continued to secrete an mAb reactive with both DNA and ENA. This cell line has now been cultured intermittently for over 3 yr without loss of reactivity. The property of dual DNA and RTE reactivity by the mAb has not been altered by continued culture or by limiting-dilution cloning of the cell line (data not shown).

The W8 human mAb next was tested for its reactivity with fixed HEp-2 cells and with Crithidia luciliae organisms in indirect immunofluorescent assays. Strong staining of HEp-2 cell nucleoli was demonstrated (Fig. 2A), whereas nucleoplasmic immunofluorescence was negligible, and cytoplasmic reactivity minimal. When present, cytoplasmic staining varied from preparation to preparation and within certain cells of an individual fixed-cell preparation. Reactivity with Crithidia kinetoplasts was not detected, even after overnight incubation with the mAb.

Thus, the staining patterns and the ELISA binding data of the W8 mAb were similar to those mentioned above for a portion of the polyclonal antibodies from the original SLE donor's serum, i.e., reactivity with both single-stranded DNA and an extractable nuclear antigen in solid-phase enzyme immunoassay and with nucleoli by immunofluorescent assay.

Partial Characterization of the Autoantigen(s) Bound by the W8 mAb. In an attempt to define the molecular characteristics of the antigen(s) recognized by the W8 mAb, fixed HEp-2 cells were treated with either DNase, RNase, or trypsin before staining with the mAb (Fig. 2). Fig. 2A illustrates the typical nucleolar staining pattern of the HEp-2 cells without enzyme digestion. Pretreatment of the cells with DNase failed to alter W8 binding to the nucleolus (Fig. 2B). However, pretreatment of the HEp-2 cells with RNase (Fig. 2C) or trypsin (not shown) completely eliminated the immunofluorescent staining. As men-
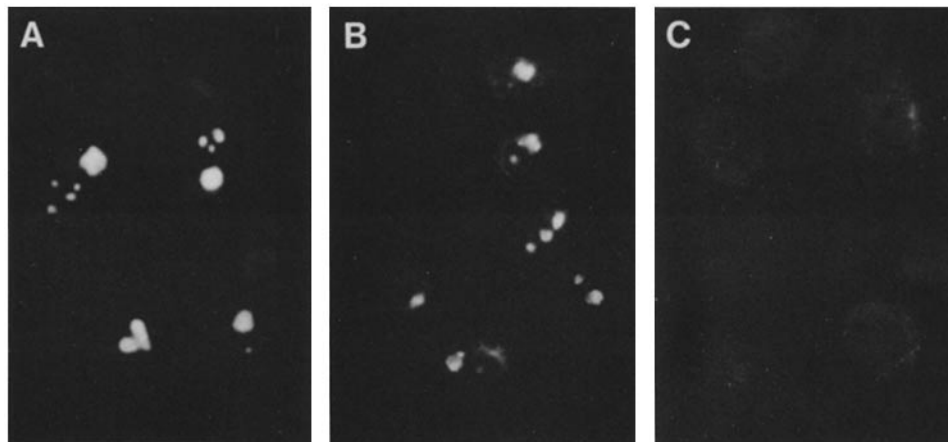
FIGURE 2. Indirect immunofluorescent staining of fixed HEp-2 cells using the W8 mAb. A, staining performed on untreated HEp-2 cells; B, staining performed after in situ DNase treatment of HEp-2 cells; C, staining performed after RNase treatment of HEp-2 cells. Original magnification × 400.

tioned above, the extractable nuclear antigens in the RTE preparation, with which the patient's serum autoantibodies reacted, had been shown previously to be RNase sensitive. As was the case for the W8 mAb, the nucleolar staining of the serum autoantibodies similarly was found to disappear with RNase digestion of the HEp-2 cells (not shown).

Because the nucleolus is transcriptionally highly active, attempts were made to determine if the antigen bound by W8 was involved in ribosomal RNA synthesis and possibly associated with the transcriptional units of the ribosome. This was evaluated by using the two antimetabolites, DRB and actinomycin D. DRB has been shown to cause nucleolar dispersal or fragmentation while inhibiting messenger but not ribosomal RNA transcription (31, 32). Appropriate doses of actinomycin D suppress ribosomal RNA synthesis without causing nucleolar dispersal (33).

When HEp-2 cells were incubated with DRB for 6 h and then stained with the W8 mAb, the usual intact nucleolar pattern was replaced by a series of distinct immunofluorescent dots (Fig. 3 B). 2 h of actinomycin D treatment resulted in a more dense condensation of the nucleolus, without an alteration of immunofluorescent staining (not shown).

Studies by others (34) have demonstrated that antibodies to RNA polymerase I stain the DRB-dispersed nucleolus in a pattern characteristic of the "necklaces" seen when DRB induces unraveling of the nucleolus (31). Since RNA polymerase I has been shown to be the target of autoantibodies in certain autoimmune conditions (35), an experiment was performed to compare the immunofluorescent staining pattern of rabbit antibodies against RNA polymerase I (36) with that of the human mAb W8. This was done by indirect immunofluorescence using a rhodamine-conjugated goat anti–rabbit antibody preparation and a fluorescein-conjugated goat anti–human antibody preparation. As depicted in Fig. 4, the rabbit antibodies to RNA polymerase I gave the typical, distinctive necklace immunofluorescent configuration (Fig. 4 A), whereas staining of the
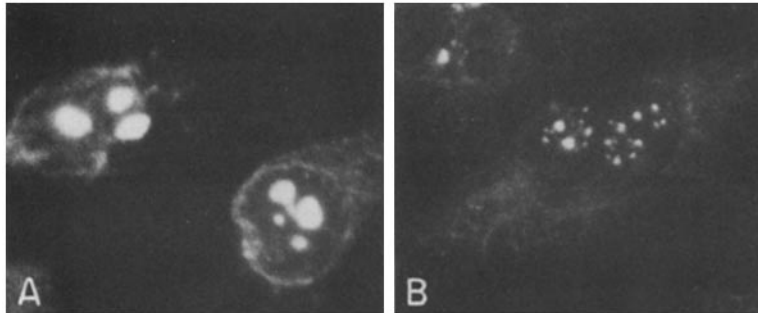
FIGURE 3. Indirect immunofluorescent staining of fixed HEp-2 cells that were grown in standard culture medium (A) or in medium supplemented by DRB (25 μg/ml) for 6 h. Original magnification × 400.

same cell with the W8 mAb (Fig. 4 B) yielded large dot-like staining that did not appear to colocalize with the smaller dots of the anti–RNA polymerase I necklace.

Comparison of the W8-extractable Nuclear Antigen with Other Known Autoantigens. The antigenic specificity recognized by the W8 mAb was compared with other previously defined reactivities in two ways. First, the extractable nuclear antigens contained in the rabbit thymus extract were subfractionated by anion-exchange chromatography and the fractions were reacted with the mAb or control serum antibodies. Because the W8 mAb recognized an extractable nuclear antigen, anti-Sm and anti-RNP autoantibodies, the first described ENA systems (37, 38), were used as reference antibodies.

Fig. 5 illustrates the pattern of binding of (a) polyclonal anti-Sm or anti-RNP reference antibodies, (b) the patient's serum and, (c) the W8 mAb to discrete fractions of RTE that were isolated by gradient (50–500 mM) NaCl elution of the RTE from a DE-52 column. As shown in Fig. 5 A, anti-Sm antibodies bind to multiple fractions of RTE eluted from the anion-exchange column; these fall into three broad peaks (I, II, III). In contrast, anti-RNP antibodies bind only those fractions in peak II. However, as illustrated in Fig. 5, B and C, the patient's serum antibodies and the W8 mAb, respectively, do not show binding patterns analogous to those observed with the anti-Sm or RNP reference autoantibodies. Rather, these antibodies bind with increasing avidity to the more highly negatively charged RTE protein fractions, suggesting that the patient's serum antibodies and the W8 mAb are interacting with acidic residues which are capable of binding relatively tightly to the anion exchanger, and which are distinct from the Sm and RNP autoantigens.

A more direct comparison between the structure of the W8 antigen and other known autoantigens was made by SDS-PAGE analysis of [^{35}S]methionine-labeled cell extracts of K562 cells immunoprecipitated by either: (a) W8, (b) the mAb from the parental fusion partner, 0467.3, (c) mAb W9 (another DNA-reactive human mAb derived from the same fusion as W8), (d) normal human serum, (e) the original patient's serum, and (f) serum from a patient with known anti-Sm antibodies (Fig. 6).

As shown previously (39), the anti-Sm serum (Fig. 6, A and B) immunoprecipitated eight major bands with molecular masses ranging from 32 to 9 kD.
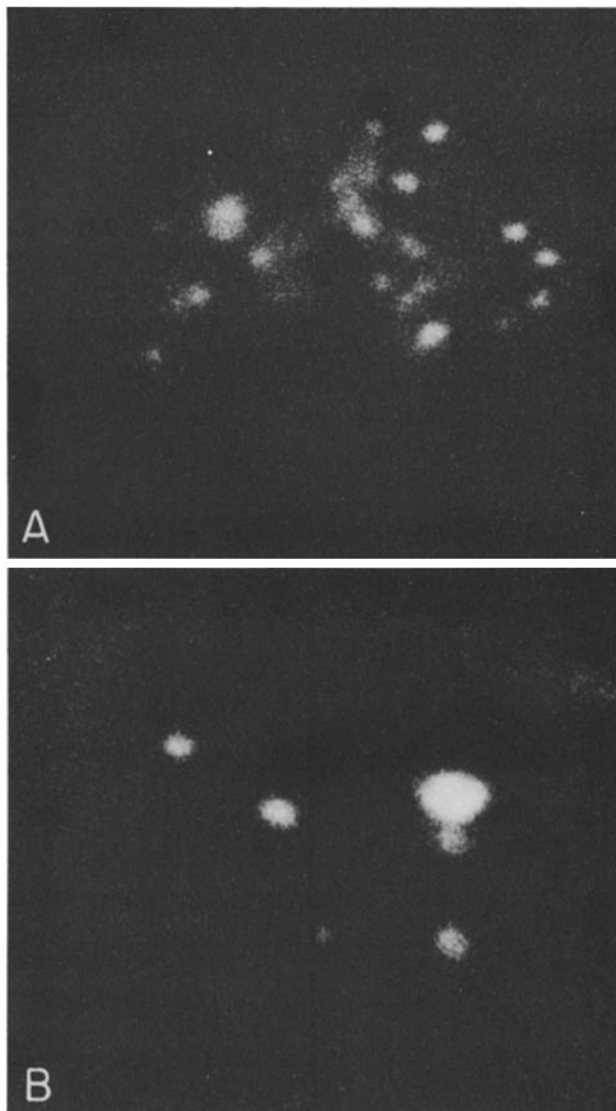
FIGURE 4. Comparison of indirect immunofluorescent staining patterns of HEp-2 cells incubated with either rabbit anti–RNA polymerase I antibodies (*A*) or human mAb W8 (*B*). HEp-2 cells were grown for 48 h on glass coverslips, washed, and then cultured for another 6 h in DRB (25 μg/ml). Rabbit antibodies to RNA polymerase I were incubated for 1 h, washed, and then counterstained with rhodamine-conjugated goat anti–rabbit antibodies at 40 μg/ml; human mAb W8 was incubated with fixed HEp-2 cells overnight at 4°C and then counterstained with FITC-conjugated goat anti–human antibodies at 40 μg/ml. *A* and *B* represent the same cell nucleus stained with these two antibody systems. Original magnification × 900.

However, as shown, the W8 mAb immunoprecipitated two major bands with molecular masses of 17 and 18 kD; bands in the 32–50 kD range also were identified, although their appearance varied from experiment to experiment and their significance is unclear at this time. Culture supernatant from the parental
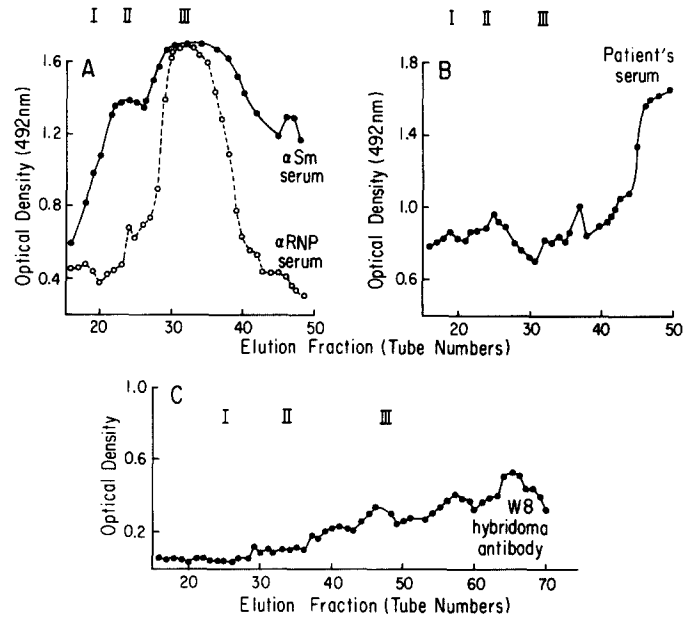
FIGURE 5. Comparison of binding patterns of polyclonal anti-Sm and anti-RNP reference antibodies (A), of the patient DW's serum (B), and the W8 mAb (C), to isolated fractions of RTE. RTE fractions were prepared as described (25) by eluting the crude, bound antigens from a DE-52 column with a 200 ml linear gradient of 50–500 mM NaCl in 10 mM sodium phosphate (pH 7.5). 50-μl samples of individual fractions were then bound to enzyme immunoassay plate wells and reacted with the various antibody preparations. Absorbance at 492 nm of each fraction is shown.

0467.3 cell line and from the W9 culture (Fig. 6 A) failed to immunoprecipitate the 17 and 18 kD bands, even after prolonged autoradiographic exposure.

Fig. 6 B demonstrates that the patient's serum antibodies immunoprecipitated several proteins, including two proteins with molecular masses similar to those immunoprecipitated by the W8 mAb (Fig. 6 B, Pt serum), indicating that the patient's serum autoantibodies and the mAb recognize the same proteins. In other experiments, immunoprecipitation analyses of [^{32}P]orthophosphoric acid–labeled cell extracts failed to demonstrate coprecipitation of labeled nucleolar RNA species including U3, 8-2, and 7-2. RNAs (data not shown).

Discussion

A human B cell line (W8) was derived that secretes an IgM mAb reactive with DNA and an ENA in enzyme immunoassay, and with nucleoli in a fluorescent ANA assay. This multiple reactivity was a function of a single antibody from a single immortalized B cell; prolonged culture and limiting-dilution cloning procedures failed to alter the data. The non-DNA antigenic moiety recognized by the W8 mAb was expressed in the nucleolus of HEp-2 cells grown for 48 h in vitro, and was sensitive to in situ enzymatic digestion with trypsin and RNase, but not DNase (Fig. 2). In addition, this mAb bound to proteins that required a high concentration of salt for displacement from a DEAE-cellulose column (Fig. 5), and therefore probably represent molecules with high concentrations of
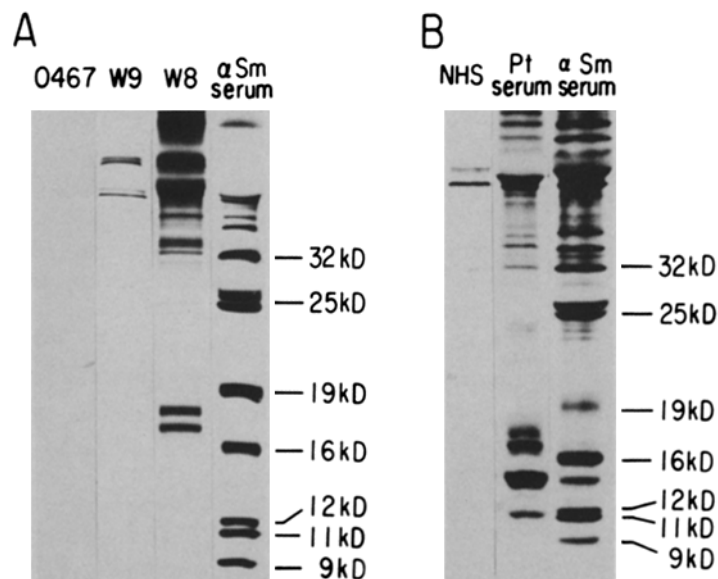
FIGURE 6. SDS-PAGE analyses of radiolabeled cellular proteins immunoprecipitated by various antibody preparations. A (left to right): 0467.3 culture supernatant, W9 mAb, W8 mAb, anti-Sm reference autoantibody containing serum. B, normal human serum, DW patient serum, anti-Sm reference antibody serum.

negative charge. Several other autoantigens are known to be negatively charged, either because of the presence of acidic residues on the protein moieties themselves or because of the intimate association of small RNA molecules with the protein moieties (40, 41). However, attempts to determine if the latter is the case for the antigen recognized by the W8 mAb have been inconclusive (see below).

In addition, attempts were made to determine whether the nucleolar antigen recognized by W8 was associated with the active transcriptional units of the nucleolus. For these experiments, the halogenated ribofuranosylbenzimidazole, DRB, was used to unravel the bound nucleolus into "nucleolar necklaces" (31), which others have suggested correspond to linear arrays of active ribosomal RNA genes and transcriptional units (34, 42). In DRB-treated HEp-2 cells, the W8 mAb appears to bind large dot-like fragments released from the nucleolus (Fig. 3B). However, it is unlikely that W8 binds to intact RNA polymerase I or to active transcriptional units, because the characteristic necklace configuration of immunofluorescence seen with antibodies to RNA polymerase I (Fig. 4A) was not visualized (Fig. 4B) in DRB-treated cells. This lack of colocalization of RNA polymerase I and W8 staining, after DRB treatment, is similar to that shown previously (34) for the S1 antigen, a protein that is present in preribosomal particles and is randomly distributed throughout the nucleus after drug exposure. It is possible that the W8 antigen, like S1, is not an epitope of the transcriptional machinery but of transcriptional products, which are dispersed separately into the cell nucleus after DRB exposure (34). In addition, treatment of the HEp-2 cells with actinomycin D resulted in further condensation of the nucleolus and

persistent immunofluorescent staining with the W8 mAb, indicating that the antigen is still expressed when ribosomal RNA synthesis is inhibited, a finding that further distinguishes the antigen bound by W8 from RNA polymerase I. Thus the W8 antigen appears to be a NaCl-extractable protein or ribonucleoprotein with acidic domains that is localized primarily in the nucleolus of HEp-2 cells but is distinct from those antigens associated with the transcriptional units of active ribosomal RNA genes.

Immunoprecipitation and SDS-PAGE analyses of [^{35}S]methionine-labeled cellular proteins have provided preliminary information regarding the molecular nature of the proteins bound by the W8 mAb. The W8 mAb immunoprecipitated at least two polypeptides with molecular masses of 17 and 18 kD (Fig. 6). Higher molecular mass bands ranging from ~35 to 50 kD have appeared at times in W8 immunoprecipitates; the present studies cannot rule out the possibility that certain of these proteins may be immunoprecipitated specifically or are coprecipitated by the W8 mAb. This is relevant because recent studies (43, 44) have demonstrated that certain SLE sera contain autoantibodies to ribosomal proteins of 38, 19, and 17 kD when analyzed by SDS-PAGE and western blotting of isolated ribosomal proteins. These proteins (P$_0$, P$_1$, P$_2$) appear to be analogous to those described in lower eukaryotic species, yeast, and bacteria (45, 46), and are acidic molecules that are part of a soluble protein–nucleic acid complex (44). Because the W8 antigen also is an RNase-sensitive, acidic protein composed of at least two chains of 17 and 18 kD, the W8 and P systems may be related. Although this possibility requires further evaluation, there is evidence that this may not be the case. First, the W8 antigen is localized primarily to the nucleolus in 48-h cultures of HEp-2 cells, whereas the P proteins are detected by SLE serum antibodies primarily in the cytoplasm (44, 47–50). Second, the molecular masses of these two sets of proteins, albeit similar, are not identical. In this regard, it should be noted that a murine mAb, prepared against isolated nucleoli and reactive with cytoplasmic P proteins, has reactivity with a nucleolar, monomeric (~38 kD) form of the P$_1$/P$_2$ proteins (51). Finally, western blotting analysis of the reactivity of the W8 mAb and the original patient's serum antibodies with isolated ribosomal proteins failed to demonstrate reactivity of either set of antibodies with the P proteins, although some reactivity of the patient's IgM serum antibodies with other ribosomal proteins was noted (K. Elkon, unpublished observations). Further studies will be necessary to determine if the W8 antigen represents a distinct antigenic system or rather a nucleolar processing variant of the cytoplasmic P proteins.

Whereas the initial characterization of the protein moieties recognized by the W8 mAb has been helpful, attempts to define a ^{32}P-labeled RNA species attached to the W8 proteins have been unsuccessful to date. Although the bulk of nucleolar RNA is preribosomal and ribosomal RNA, certain small nuclear species, in particular U3, 7-1, 7-2, and 8 S RNAs accumulate in the nucleolus in association with pre–ribosomal RNA and ribosomal RNA. Previous studies have suggested the existence of autoantibodies that coprecipitate U3 (37), or 7-2 and 8-2 (52, 53), nucleolar small RNAs. The protein moieties of these small nucleolar ribonucleoproteins, which by analogy with other ANA systems are probably the targets of the autoantibodies, have not been identified to date. However, it seems

unlikely that the antigen recognized by the W8 mAb is a component of these three types of smaller nucleolar ribonucleoproteins because U3, 7-2, and 8-2 S RNAs were not coprecipitated by the mAb or by the patient's serum, while in the same experiment, reference antibodies to To/Th (52, 53) coprecipitated 7-2 and 8-2 RNA (data not shown). The reason(s) for these negative data are not yet clear. One possibility is that the antigen recognized by the mAb binds to a large RNA species (e.g., rRNA precursor) that is not efficiently immunoprecipitated, as has been noted in other systems (54). A second possibility is that the mAb recognizes a conformational determinant formed by protein and nucleic acid moieties that are in close proximity within the nucleolus but not permanently associated; in such a situation the noncovalently linked complex might not immunoprecipitate well. Finally, the affinity of the IgM W8 mAb may be involved. In this regard, our studies (55) and those of others (14, 17) have shown that human IgM mAb that react with DNA and other cellular constituents are frequently of low antigen-binding activity and therefore may be difficult to use for immunoprecipitation analyses. Studies to distinguish between these possibilities are in progress.

The multiple antigenic reactivities of the W8 mAb are notable. This IgM mAb binds quite well to ssDNA and the nucleolar ENA described above. The phenomenon of dual reactivity of an mAb with DNA and other cellular structures has been reported previously (55–58). In humans, IgM mAb reactive with DNA have been shown to also bind to and immunoprecipitate the intermediate filament, vimentin (56); in mice, an mAb has been reported that is reactive with DNA and Sm (58). The frequency at which the W8 and other crossreactive antibody specificities occur in the SLE population is unclear at this time. However, a retrospective review of SDS-PAGE analyses of labeled cellular proteins immunoprecipitated by a panel of SLE sera suggest that the W8 specificity is not common.

Finally, if the W8 hybridoma cell is an immortalization of an autoantibody-producing clone, as our data would suggest, then it is intriguing to postulate that the apparently distinct serum autoantibody populations directed at ssDNA, ENA, and nucleoli are actually the manifestation of the same antibody population that is represented by the W8 mAb. This hypothesis is currently under investigation. In any event, it is clear that human mAb obtained by immortalization of autoreactive B cells are valuable probes for examining autoantigenic structures shared by different molecules, such as nucleic acids and proteins. The multiple reactivities displayed by the W8 and other such mAb might be illustrative of the reactivities of other naturally occurring autoantibodies seen in normal and diseased individuals.

Summary

B lymphocytes from a patient with systemic lupus erythematosus (SLE) and several circulating autoantibodies (including antinucleolar antibodies) were immortalized by fusion with a hypoxanthine/guanine phosphoribosyl transferase (HGPRT)-deficient human B cell line. Multiple human monoclonal antibodies (mAb) were obtained which, in solid-phase enzyme immunoassay, were reactive with DNA. One mAb was of special interest because it reacted strongly with

both single-stranded DNA and an extractable nuclear antigen found in rabbit thymus extract (RTE). In an immunofluorescent assay using fixed human cells, the latter mAb also bound predominantly to cell nucleoli. A combination of enzyme digestion and metabolic inhibitor studies of the target cells in this immunofluorescent assay suggested that the antigen(s) bound by the mAb was an RNA-associated protein or a ribonucleoprotein that is distinct from intact RNA polymerase I and not associated with the transcriptional units of the nucleolus. In other experiments, using fractions of RTE isolated by ion-exchange chromatography, the antigens bound by the mAb were shown to be highly negatively charged molecules. Immunoprecipitation and SDS-PAGE analyses of labeled cell extracts bound by the mAb revealed a doublet of 17 and 18 kD.

Since the original patient's serum autoantibodies also bound to both an RNase-sensitive, acidic, extractable nuclear antigen and to nucleoli, and immunoprecip-itated proteins of similar molecular masses in SDS-PAGE, it appears that the described mAb is a product of an immortalized autoantibody-producing B cell clone from the SLE patient's peripheral blood. This mAb probably defines a novel RNA-associated autoantigen residing predominantly in the nucleolus or, less likely, a variant of either RNA polymerase I or the ribosomal autoantigens (P proteins).

References

1. Lerner, E. A., M. R. Lerner, C. A. Janeway, and J. A. Steitz. 1981. Monoclonal antibodies to nucleic acid-containing cellular constituents: Probes for molecular biology and autoimmune disease. *Proc. Natl. Acad. Sci. USA.* 78:2737.
2. Pisetsky, D. S., and E. A. Lerner. 1982. Idiotypic analysis of monoclonal anti-Sm antibody. *J. Immunol.* 129:1489.
3. Billings, P. B., R. W. Allen, F. C. Jensen, and S. O. Hoch. Anti-RNP monoclonal antibodies derived from a mouse strain with lupus-like autoimmunity. *J. Immunol.* 126:1176.
4. Pisetsky, D. S., D. M. Salistad, and J. C. Chambers. 1984. Characterization and idiotypic analysis of an anti-RNP monoclonal antibody. *Clin. Exp. Immunol.* 56:593.
5. Andrzejewski, C., Jr., B. D. Stollar, T. M. Lalor, and R. S. Schwartz. Hybridoma autoantibodies to DNA. *J. Immunol.* 124:1499.
6. Hahn, B. H., F. Ebling, S. Freeman, B. Clevinger, and J. Davie. 1980. Production of monoclonal murine antibodies to DNA by somatic cell hybrids. *Arthritis Rheum.* 23:942.
7. Tron, F., D. Charron, J. F. Bach, and N. Talal. 1980. Establishment and characterization of a murine hybridoma secreting monoclonal anti-DNA autoantibody. *J. Immunol.* 125:2805.
8. Klotz, J. L., M. L. Phillips, M. M. Miller, and R. L. Teplitz. 1981. Monoclonal antibody production by hybrid cell lines. *Clin. Immunol. Immunopathol.* 18:368.

9. Lee, J. S., J. R. Lewis, A. R. Morgan, T. R. Mosmann, and B. Singh. 1981. Monoclonal antibodies showing sequence specificity in their interaction with single stranded DNAs. *Nucleic Acids Res.* 9:1707.

10. Pisetsky, D. S., and S. A. Caster. 1982. Binding specificity of a monoclonal anti-DNA antibody. *Mol. Immunol.* 19:645.

11. Ballard, D. W., and E. W. Voss. 1982. Monoclonal murine anti–nucleic acid antibody with double-stranded specificity. *Mol. Immunol.* 19:793.

12. Kardost, R. R. P., P. A. Billing, and E. W. Voss. 1982. Generation and characterization of three monoclonal murine nucleotide binding anti-ssDNA autoantibodies. *Mol. Immunol.* 19:963.

13. Eilat, D., R. Asofsky, and R. Laskov. 1980. A hybridoma from an autoimmune NZB/NZW mouse producing monoclonal antibody to ribosomal RNA. *J. Immunol.* 124:766.

14. Schoenfeld, Y., S. C. Hsu-Lin, J. E. Gabriels, L. E. Silberstein, B. C. Furie, B. Furie, B. D. Stollar, and R. S. Schwartz. 1982. Production of autoantibodies by human-human hybridomas. *J. Clin. Invest.* 70:205.

15. Littman, B. H., A. V. Muchmore, A. D. Steinberg, and W. C. Greene. 1983. Monoclonal autoantibody secretion by human-human hybridomas. *J. Clin. Invest.* 72:1987.

16. Schoenfeld, Y., J. Rauch, H. Massicotte, S. K. Datta, J. Andre-Schwartz, B. D. Stollar, and R. S. Schwartz. 1983. Polyspecificity of monoclonal lupus autoantibodies produced by human-human hybridomas. *N. Engl. J. Med.* 308:414.

17. Hoch, S., and J. Schwaber. 1986. Specificity analysis of human anti-DNA antibodies. *J. Immunol.* 136:892.

18. Satoh, J., B. S. Prabhakar, M. V. Haspel, F. Ginsberg-Fellner, and A. L. Notkins. 1983. Human monoclonal autoantibodies that react with multiple endocrine organs. *N. Engl. J. Med.* 309:217.

19. Chiorazzi, N., and W. Reeves. 1985. A human monoclonal antibody reactive with DNA, extractable nuclear antigen, and nucleoli. *Arthritis Rheum.* 28:S50.

20. Hoffman, T., and H. G. Kunkel. 1976. The Erosette test. *In* In Vitro Methods in Cell-mediated and Tumor Immunity. B. R. Bloom and J. R. David, editors. Academic Press, New York. 71–82.

21. Chiorazzi, N., R. L. Wasserman, and H. G. Kunkel. 1982. Use of Epstein-Barr virus-transformed B cell lines for the generation of immunoglobulin-producing human B cell hybridomas. *J. Exp. Med.* 156:930.

22. Moore, A. E., L. Sabachewsky, and H. W. Toolan. 1955. Culture characteristics of four permanent lines of human cancer cells. *Cancer Res.* 15:598.

23. Eaton, R. B., G. Schneider, and P. H. Schur. 1983. Enzyme immunoassay for antibodies to native DNA. Specificity and quality of antibodies. *Arthritis Rheum.* 26:52.

24. Giallongo, A., L. Kochoumian, and T. P. King. 1982. Enzyme and radioimmunoassays for specific murine IgE and IgG with different solid phase immunosorbants. *J. Immunol. Methods.* 52:379.

25. Reeves, W. H., D. E. Fisher, R. G. Lahita, and H. G. Kunkel. 1985. Autoimmune sera reactive with Sm antigen contain high levels of RNP-like antibodies. *J. Clin. Invest.* 75:580.

26. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pg. 451.

27. Reeves, W. H. 1985. Use of monoclonal antibodies for the characterization of novel DNA-binding proteins recognized by human autoimmune sera. *J. Exp. Med.* 161:18.

28. Fisher, D. E., W. H. Reeves, G. E. Conner, G. Blobel, and H. G. Kunkel. 1983.

Synthesis and assembly of human small nuclear ribonucleoproteins generated by cell free translation. *Proc. Natl. Acad. Sci. USA.* 80:6356.

29. Bonner, W., and R. Laskey. 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46:83.

30. Tan, E. M., and C. Peebles. 1980. Quantitation of antibodies to Sm antigen and nuclear ribonucleo protein by hemagglutination. *In* Manual of Clinical Immunology. N. R. Rose and H. Friedman, editors. American Society for Microbiology Press, Washington, DC. pp. 866–871.

31. Granick, D. 1975. Nucleolar necklaces in chick embryo fibroblast cells. I. Formation of necklaces by dichlororibobenzimidazole and other adenosine analogues that decrease RNA synthesis and degrade preribosomes. *J. Cell Biol.* 65:398.

32. Tamm, I., P. B. Sehgal, R. A. Lamb, and A. R. Goldberg. 1984. Halogenated ribofuranosylbenzimidazoles. *In* Antiviral Drugs and Interferon: The Molecular Basis of Their Activity. Martinus Nijhoff Publishing, Boston, MA. pp. 203–252.

33. Phillips, S. G., and D. M. Phillips. 1971. Nucleoli of diploid cell strains. Their normal ultrastructure and the effects of toyocamycin and actinomycin D. *J. Cell Biol.* 49:785.

34. Scheer, U., B. Hugle, R. Hazan, and K. M. Rose. 1984. Drug-induced dispersal of transcribed rRNA genes and transcriptional products: Immunolocalization and silver staining of different nucleolar components in rat cells treated with 5,6,-dichloro-β-D-ribofuranosylbenzimidazole. *J. Cell Biol.* 99:672.

35. Stetler, D. A., K. M. Rose, M. E. Wenger, C. M. Berlin, and S. T. Jacob. 1982. Antibodies to distinct polypeptides of RNA polymerase I in sera from patients with rheumatic autoimmune disease. *Proc. Natl. Acad. Sci. USA.* 79:7499.

36. Scheer, U., G. Lanfranchi, K. M. Rose, W. W. Franke, and N. R. Ringertz. 1983. Migration of rat RNA polymerase I into chick erythrocyte nuclei undergoing reactivation in chick-rat heterokaryons. *J. Cell Biol.* 97:1641.

37. Tan, E. M., and H. G. Kunkel. 1966. Characteristics of a soluble nuclear antigen precipitating with sera of patients with systemic lupus erythematosus. *J. Immunol.* 96:464.

38. Sharp, G. C., W. S. Irvin, R. L. LaRoque, C. Velez, V. Daly, A. D. Kaiser, and H. R. Holman. 1971. Associations of autoantibodies to different nuclear antigens with clinical patterns of rheumatic disease and responsiveness to therapy. *J. Clin. Invest.* 50:350.

39. Conner, G. E., D. Nelson, R. Wisniewolski, R. G. Lahita, G. Blobel, and H. G. Kunkel. 1982. Protein antigens of the RNA-protein complexes detected by anti-Sm and anti-RNP antibodies found in the serum of patients with systemic lupus erythematosus and related disorders. *J. Exp. Med.* 156:1475.

40. Hinterberger, M., I. Petterson, and J. A. Steitz. 1983. Isolation of small nuclear ribonucleoproteins containing U1, U2, U4, U5, and U6 RNAs. *J. Biol. Chem.* 258:2604.

41. Kinlaw, C. S., B. L. Robberson, and S. M. Berget. 1983. Fractionation and characterization of human small nuclear ribonucleoproteins containing U1 and U2 RNAs. *J. Biol. Chem.* 258:7181.

42. Scheer, U., and K. M. Rose. 1984. Localization of RNA polymerase I in interphase cells and mitotic chromosomes by light and electron microscopic immunocytochemistry. *Proc. Natl. Acad. Sci. USA.* 81:1431.

43. Francoeur, A. M., C. L. Peebles, K. J. Heckman, J. C. Lee, and E. M. Tan. 1985. Identification of ribosomal protein autoantigens. *J. Immunol.* 135:2378.

44. Elkon, K. B., A. P. Parnassa, and C. L. Foster. 1985. Lupus autoantibodies target ribosomal P proteins. *J. Exp. Med.* 162:459.

45. Vidales, F. J., F. Sanchez-Madrid, and J. P. G. Ballesta. 1981. The acidic proteins of eucharyotic ribosomes. A comparative study. *Biochim. Biophys. Acta.* 656:28.

46. Sanchez-Madrid, F., R. Reyes, P. Conde, and J. F. Ballesta. 1979. Acidic ribosomal proteins from eukaryotic cells. Effect on ribosomal functions. *Eur. J. Biochem.* 98:409.

47. Homberg, J. C., M. Rizzetto, and D. Doniach. 1974. Ribosomal antibodies detected by immunofluorescence in systemic lupus erythematosus and other collagenoses. *Clin. Exp. Immunol.* 4:45.

48. Miyachi, K., and E. M. Tan. 1979. Antibodies reacting with ribosomal nucleoprotein in connective tissue diseases. *Arthritis Rheum.* 22:87.

49. Koffler, D., T. E. Miller, and R. G. Lahita. 1979. Studies on the specificity and clinical correlation of antiribosomal antibodies in systemic lupus erythematosus sera. *Arthritis Rheum.* 22:463–470.

50. Meroni, P. L., G. DeBartolo, W. Barcellini, P. S. Riboldi, R. Basile, C. Betterle, and C. Zanussi. 1984. Anti-ribosomal ribonucleoprotein autoantibodies in systemic lupus erythematosus. *J. Clin. Immunol.* 4:45.

51. Towbin, H., H-P. Ramjoue, H. Kuster, D. Liverani, and J. Gordon. 1982. Monoclonal antibodies against eucaryotic ribosomes. Use to characterize a ribosomal protein not previously identified and antigenically related to the acidic phosphoproteins P1/P2. *J. Biol. Chem.* 257:12709.

52. Reddy, R., E. M. Tan, D. Henning, K. Nohga, and H. Busch. 1983. Detection of a nucleolar 7-2 ribonucleoprotein and a cytoplasmic 8-2 ribonucleoprotein with auto-antibodies from patients with Scleroderma. *J. Biol. Chem.* 258:1383.

53. Hashimoto, C., and J. Steitz. 1983. Sequential association of nucleolar 7-2 RNA with two different autoantigens. *J. Biol. Chem.* 258:1379.

54. Matter, L., K. Schopfer, J. A. Wilhelm, T. Nyffenegger, R. F. Parisot, and E. M. DeRobertis. 1982. Molecular characterization of ribonucleoprotein antigens bound by antinuclear antibodies: A diagnostic evaluation. *Arthritis Rheum.* 25:1278.

55. Chiorazzi, N., R. D. Bigler, R. Taylor, R. G. Lahita, and W. H. Reeves. 1986. Antigen-binding properties of a subset of human IgM monoclonal antibodies reactive with DNA. *Arthritis Rheum.* 29:S71.

56. Andre-Schwartz, J., S. K. Datta, Y. Schoenfeld, D. A. Isenberg, D. Stollar, and R. S. Schwartz. 1984. Binding of cytoskeletal proteins by monoclonal anti-DNA lupus autoantibodies. *Clin. Immunol. Immunopathol.* 31:261.

57. Jacob, L., F. Tron, J. F. Bach, and D. Louvard. 1984. A monoclonal anti-DNA antibody also binds to cell-surface protein(s). *Proc. Natl. Acad. Sci. USA.* 81:3843.

58. Pisetsky, D. S., S. O. Hoch, C. L. Klatt, M. A. O'Donnell, and J. D. Keene. 1985. Specificity and idiotypic analysis of a monoclonal anti-Sm antibody with anti-DNA activity. *J. Immunol.* 135:4080.