Identification of Autoantibodies to RNA Polymerase II

Occurrence in Systemic Sclerosis and Association with Autoantibodies to RNA Polymerases I and III

Michito Hirakata,* Yutaka Okano,‡ Uttam Pati,§ Akira Suwa,* Thomas A. Medsger, Jr.,‡ John A. Hardin,§ and Joe Craft*
**Section of Rheumatology, Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06510; ‡Division of Rheumatology and Clinical Immunology, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261; and §Department of Medicine, Medical College of Georgia, Augusta, Georgia 30912-3100*

Abstract

In this study, autoantibodies to RNA polymerase II from sera of patients with systemic sclerosis have been identified and characterized. These antibodies immunoprecipitated polypeptides of 220 kD (IIA) and 145 kD (IIC), the two largest subunits of RNA polymerase II, and bound both subunits in immunoblots. These polypeptides were immunoprecipitated by the anti–RNA polymerase II monoclonal antibody 8WG16, which recognizes the carboxyl-terminal domain of the 220-kD subunit, and their identity to the proteins bound by human sera was confirmed in immunodepletion studies. Sera with anti–RNA polymerase II antibodies also immunoprecipitated proteins that were consistent with components of RNA polymerases I and III. In vitro transcription experiments showed that the human antibodies were an effective inhibitor of RNA polymerase II activity. In indirect immunofluorescence studies, anti–RNA polymerase II autoantibodies stained the nucleoplasm, as expected from the known location of RNA polymerase II, and colocalized with the anti–RNA polymerase II monoclonal antibody. The human sera also stained the nucleolus, the location of RNA polymerase I. From a clinical perspective, these antibodies were found in 13 of 278 patients with systemic sclerosis, including 10 with diffuse and three with limited cutaneous disease, but were not detected in sera from patients with other connective tissue diseases and from normal controls. We conclude that anti–RNA polymerase II antibodies are specific to patients with systemic sclerosis, and that they are apparently associated with antibodies to RNA polymerases I and III. These autoantibodies may be useful diagnostically and as a probe for further studies of the biological function of RNA polymerases. (*J. Clin. Invest.* 1993. 91:2665–2672.) Key words: RNA polymerases • autoantibodies • antinuclear antibodies • systemic sclerosis • autoimmunity

Introduction

The three RNA polymerases (RNAP)[1] catalyze the transcription of different sets of genes into RNA. RNAP I synthesizes ribosomal RNA precursors in nucleoli. RNAP II synthesizes the precursors of mRNAs and most of the small nuclear RNAs which are found in ribonucleoprotein (RNP) particles that mediate pre-mRNA splicing. RNAP III synthesizes small RNAs, including 5S ribosomal RNAs and transfer RNAs. These multimeric enzymes are comprised of 8–14 polypeptide subunits, depending upon the species from which they are isolated (1, 2); however, the details of their complex structures are still being defined.

In mammalian cells, RNAP II contains two large polypeptides (220 kD and 145 kD) that are highly conserved across species, and at least six smaller subunits (1, 2). Several of the latter may be shared among RNAP I, II, and III, whereas the larger two subunits are unique to RNAP II (1–5). The largest 220-kD polypeptide of RNAP II migrates as a phosphorylated subunit of 240 kD, a nonphosphorylated subunit of 220 kD, and a proteolytic derivative of 180 kD, referred to as IIO, IIA, and IIB, respectively, when analyzed in SDS-polyacrylamide gels (4, 6–8). Recent studies have revealed that the 220-kD subunit contains a unique carboxyl-terminal domain (CTD) consisting of a Tyr-Ser-Pro-Thr-Ser-Pro-Ser heptapeptide that is repeated 52 times in mammalian cells (2, 4, 7, 9). The CTD is released by proteolysis generating the 180-kD subunit (10, 11). This sequence is highly phosphorylated, required for cell viability, and thought to mediate promoter specific transcription (12, 13). Thompson and colleagues have developed a monoclonal antibody referred to as 8WG16 (anti-CTD mAb) that reacts with this highly conserved carboxyl-terminal repeat of the 220-kD subunit (14, 15).

Some patients with the connective tissue disease systemic sclerosis (scleroderma) have been shown to produce autoantibodies that bind RNA polymerase I (16, 17). Corresponding autoantibodies to RNAP II and III have not yet been identified. In the present study, we have identified and characterized autoantibodies from sera of patients with systemic sclerosis that recognize RNAP II subunits.

Methods

Human autoimmune sera and RNAP II monoclonal antibody. Sera were obtained from 84 patients with systemic sclerosis (diffuse cutaneous systemic sclerosis, limited cutaneous systemic sclerosis, and systemic sclerosis overlap syndromes) who were identified from a review of patient records of the Section of Rheumatology, Yale University

Address correspondence to Joe Craft, M.D., Section of Rheumatology, 610 LCI, Yale University School of Medicine, Box 3333, 333 Cedar St., New Haven, CT 06510.

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1. *Abbreviations used in this paper:* CMV, cytomegalovirus; CTD, carboxyl-terminal domain; MCTD, mixed connective tissue disease; PM/DM, polymyositis or dermatomyositis; RNAP, RNA polymerase; RNP, ribonucleoprotein; snRNP, small nuclear RNP; TBS, Tris-buffered saline.

School of Medicine, and from 194 patients with systemic sclerosis who were seen at the University of Pittsburgh School of Medicine during 1986–1988. All patients seen at Yale University and 167 of the 194 patients seen at University of Pittsburgh fulfilled the preliminary criteria of the American College of Rheumatology for classification as definite systemic sclerosis (18). Sera from 217 patients with other systemic autoimmune diseases, including SLE (n = 126) (19), polymyositis/dermatomyositis (PM/DM) (n = 64) (20), and mixed connective tissue disease (MCTD) (n = 27) (21), and sera from 30 normal individuals were used as controls. A murine monoclonal antibody (8WG16; anti-CTD mAb) (14), raised against wheat germ RNAP II, which reacts with the carboxyl-terminal repeat domain of the largest subunit of RNAP II, was a kind gift from Dr. Nancy E. Thompson (McArdle Laboratory for Cancer Research, University of Wisconsin). This mAb binds the 220-kD large subunit of RNAP II in immunoblots and a synthetic heptapeptide repeat corresponding to the CTD region of this polypeptide. It also inhibits promoter-specific transcription by RNAP II (14, 15).

Indirect immunofluorescence. Human sera were diluted 1:40 in Tris-buffered saline (TBS) (150 mM NaCl, 10 mM Tris HCl, pH 7.4), and were screened by indirect immunofluorescence (22) using HEp-2 cells (Immunoconcepts, Sacramento, CA) with goat anti–human IgG fluorescein isothiocyanate conjugate (Sigma, St. Louis, MO) and goat anti–mouse IgG Texas red conjugate (Tago, Inc., Burlingame, CA). Each well was viewed at a magnification of 1,000 on an immunofluorescence microscope (Carl Zeiss, Oberkochen, Germany) to detect cytoplasmic, nuclear, and nucleolar staining.

Preparation of radiolabeled cell extracts. HeLa cells ($2–2.5 \times 10^5$ cells/ml) were radiolabeled for 14 h in deficient media, as previously described, with [^{35}S]methionine (10 μCi/ml cells) (ICN Biomedical Inc., Irvine, CA) for analysis of proteins and with [^{32}P]orthophosphate (10 μCi/ml cells) (ICN Biomedical Inc.) for RNA analysis (22, 23). Cells were collected by centrifugation, washed in TBS (10 mM Tris Cl, pH 7.5, 150 mM NaCl), and sonicated three times each for 40 s in NET-2 buffer (50 mM Tris Cl, pH 7.5, 150 mM NaCl, 0.05% Nonidet P-40) with a sonifier (Branson Sonic, Danbury, CT) at setting 3. The sonicated lysate was centrifuged at 15,000 g for 10 min to remove cellular debris.

Immunoprecipitation of radiolabeled cell extracts. Immunoprecipitation of radiolabeled cell extracts was performed as previously described (22–24). Briefly, 10 μl of patient sera was mixed with 2 mg of protein A-sepharose CL-4B (Pharmacia Inc., Piscataway, NJ) in 500 μl of immunoprecipitation buffer (IPP) (10 mM Tris Cl, pH 8.0, 500 mM NaCl, 0.1% Nonidet P-40) and incubated with end-over-end rotation (Labquake shaker; Lab Industries, Berkeley, CA) for 2 h at 4°C. The sepharose particles with adsorbed IgG were washed four times in 500 μl of IPP buffer using 10-s spins in a microfuge tube, and resuspended in 400 μl of NET-2 buffer. For protein studies, antibody-coated sepharose beads were mixed with 400 μl of [^{35}S]methionine-labeled extracts (8×10^6 cells) and rotated at 4°C for 2 h. After four washes with NET-2, the sepharose beads were resuspended in SDS sample buffer (2% SDS, 10% glycerol, 62.5 mM Tris Cl, pH 6.8, 0.005% bromophenol blue). After heating (90°C for 5 min), the proteins were fractionated on SDS-10% polyacrylamide gels, enhanced with 0.5 M sodium salicylate, and dried; labeled proteins were analyzed by autoradiography. Immunoprecipitated RNAs were analyzed as previously described (22, 23).

Antigen depletion. [^{35}S]methionine-labeled HeLa cell extracts were incubated with 20–160 μg of anti-CTD mAb bound to protein A Sepharose CL-4B for 2 h at 4°C to deplete the extracts of RNAP II. The depleted supernatant was preincubated with protein A-sepharose to absorb any antibody excess, and then used in immunoprecipitation as the antigen source for patient anti–RNAP II sera. In the reciprocal experiment, [^{35}S]methionine-labeled HeLa cell extracts were absorbed with 40 μg of IgG isolated from an anti–RNAP II human serum as described and then probed with the anti-CTD mAb. The same amount of IgG from a normal human serum and from an SLE serum with the anti-Sm specificity (25) were included as controls.

In vitro transcription from the adenovirus major late promoter and from the cytomegalovirus (CMV) immediate early promoter. Dignam extracts from HeLa cells were used for studying transcription from both these promoters (26). For transcription from the adenovirus major late promoter, the plasmid vector pBR 322 containing the 877-bp fragment with the major late promoter region (from position −680 to position +197) was prepared as previously described (27). The gel-purified fragment was used as a template in a transcription assay to generate a single 197-nucleotide transcript. Transcription from the CMV immediate early promoter (Promega, Madison, WI) was carried out according to the protocol of the manufacturer; the CMV run-off product was 363 nucleotides.

Purification of RNAP II subunits. Purification of RNA polymerase II from calf thymus was carried out by a modification of the method of Hodo and Blatti (28), except that all buffers contained 0.1% Nonidet P-40. Fractions from the phosphocellulose column containing RNAP II activity were pooled and applied to a heparin-agarose column equilibrated with buffer containing 60 mM ammonium sulfate. Active fractions were eluted with buffer containing 500 mM ammonium sulfate. The active fractions were pooled and passed through a Sephadex A-25 column. RNAP II was further purified by glycerol gradient centrifugation. RNAP II polypeptides were analyzed on gradient (7–15%) polyacrylamide gels and the 145-kD polypeptide of RNAP II was separated from the gel by an electroeluter (Bio Rad, Richmond, CA). Purification of the 220-kD polypeptide of RNAP II from HeLa cells was carried out by immunoaffinity chromatography with the anti-CTD mAb 8WG16 (14, 15). The purified 145-kD and 220-kD subunits were used as substrates for immunoblots.

Immunoblots. Immunoblots were performed by a modification of the Towbin et al. procedure (29).

Results

Immunoprecipitation. Serum samples from 278 patients with systemic sclerosis and 217 patients with other connective tissue diseases, and samples from 30 normal blood donors were tested for the presence of antibodies against RNAP II in radioimmunoprecipitation assays. An initial prototype serum Wa was identified, which immunoprecipitated polypeptides of 240, 220, and 145 kD, which correspond to known subunits of RNAP II (1, 2) (Figs. 1 and 2, lane *2*). The anti-CTD mAb 8WG16 also immunoprecipitated the same polypeptides (Figs. 1 and 2, lane *1*). It should be noted that this antibody binds to the CTD of the 220- and 240-kD subunits, and that the latter polypeptide runs as a smear on gels since it is highly phosphorylated. The 145-kD subunit is included in the immunoprecipitates formed with this mAb, since it is part of the multimeric RNAP II complex (1, 2). It should also be noted that the anti-CTD mAb immunoprecipitated the 23-kD subunit (Fig. 1, lane *1*), one of the smaller subunits shared among all three polymerases (1, 2). In contrast to the findings with the 8WG16 mAb and serum Wa, control sera and a control mAb (the Y12 anti-Sm mouse mAb) did not immunoprecipitate any of these polypeptides (Fig. 2, lanes *3–7*). The band of ~ 200 kD bound by anti-Sm antibodies that migrates slightly faster than the 220-kD subunit of RNAP II is a component of the 20S U5 snRNP (30) (Fig. 2, compare lanes *3* and *4* with lanes *1* and *2*).

Among the 278 sera from patients with systemic sclerosis, 13 produced immunoprecipitates containing these same polypeptides, as demonstrated by nine representative sera (Fig. 1, lanes *2–10*). 12 of the 13 sera (including the prototype serum Wa) also immunoprecipitated six additional polypeptides with

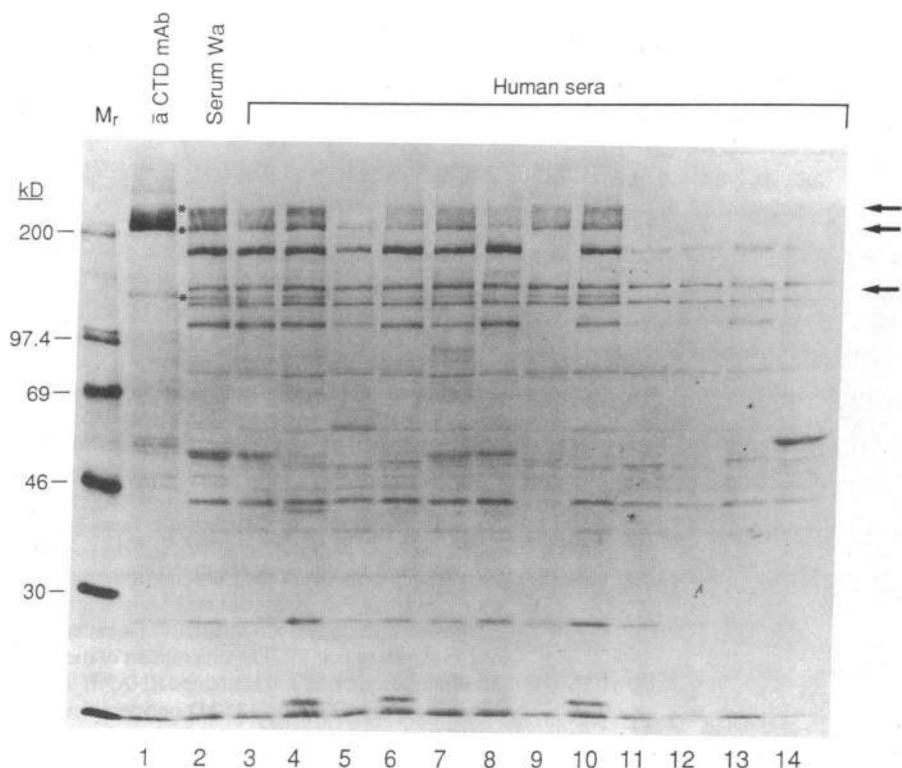


Figure 1. [^{35}S]methionine-labeled HeLa cell proteins immunoprecipitated with human sera and the anti-CTD mAb, followed by resolution in an SDS-7% polyacrylamide gel (24). The anti-CTD mAb immunoprecipitated RNAP II polypeptides of 240, 220, and 145 kD (lane *1*). The anti-RNAP II prototype serum Wa (lane *2*) and other representative sera from patients with systemic sclerosis immunoprecipitated the identical polypeptides (lanes *3–10*). These sera also immunoprecipitated polypeptides of 190, 155, 138, 126, 44, and 23 kD (lanes *2–8* and *10*), except for one serum (lane *9*), which did not react with the 190-, 126-, and 44-kD polypeptides. Sera presumably containing anti-RNAP I/RNAP III antibodies immunoprecipitated the same six polypeptides, but not those of 240, 220, and 145 kD (lanes *11–14*). M_r shows molecular weight markers; asterisks and arrows denote the 240-, 220-, and 145-kD subunits of RNAP II.

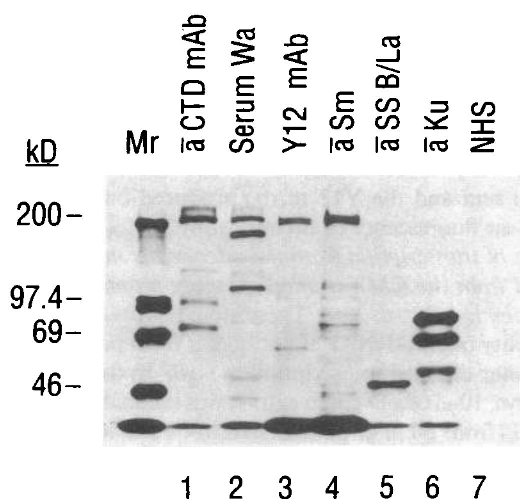


Figure 2. [^{35}S]methionine-labeled HeLa cell proteins immunoprecipitated with the anti-CTD mAb, human sera, and the Y12 anti-Sm mAb, followed by resolution in an SDS-7% polyacrylamide gel (24). The anti-CTD mAb immunoprecipitated RNAP II polypeptides of 240, 220, and 145 kD (lane *1*); the bands at ~ 100 and 70 kD represent degradation products that were not reproducibly seen in other immunoprecipitates formed with the anti-CTD mAb. The anti-RNAP II prototype serum Wa immunoprecipitated the identical polypeptides as the CTD mAb (lane *2*). The Y12 anti-Sm mAb and an anti-Sm patient serum immunoprecipitated the U5-specific doublet of 200 kD (30) and other snRNP proteins (lanes *3* and *4*). An anti-La patient serum immunoprecipitated the 48-kD La protein (lane *5*), an anti-Ku patient serum immunoprecipitated the 70- and 80-kD Ku polypeptides (lane *6*), and a normal human serum (*NHS*) did not immunoprecipitate any specific polypeptides. M_r shows molecular weight markers.

molecular masses of 190, 155, 138, 126, 44, and 23 kD, which correspond to known subunits of RNAP I and RNAP III (1) (Fig. 1, examples in lanes *2–8* and *10*). In contrast, the serum shown in lane *9* immunoprecipitated only the 155-, 138-, and 23-kD polypeptides, along with the 240-, 220-, and 145-kD subunits of RNAP II.

Sera from another 54 patients with systemic sclerosis immunoprecipitated the six smaller proteins (190, 155, 138, 126, 44, and 23 kD), without immunoprecipitation of the 240-, 220-, and 145-kD subunits of RNAP II (Fig. 1, examples in lanes *11–14*). None of the control specimens, consisting of 30 normal human sera and 217 autoimmune sera from patients with SLE, MCTD, and PM/DM were able to immunoprecipitate the RNAP II profile, or the 190-, 155-, 138-, 126-, 44-, and 23-kD proteins which, by their size, are consistent with components of RNAP I and III. When studies were carried out with HeLa cells labeled with [^{32}P]orthophosphate (22, 23), no small RNAs were specifically immunoprecipitated by 13 anti-RNAP II sera, in contrast to their ready immunoprecipitation by positive control sera (anti-Sm, anti-U1 RNP, anti-Ro, and anti-La; data not shown).

Immunodepletion studies. To confirm that the anti-CTD mAb and the putative anti-RNAP II sera identified above recognized the same cell components, extracts depleted of RNAP II by absorption with the anti-CTD mAb next were used in immunoprecipitation experiments. This mAb depleted extracts of the 240-, 220-, and 145-kD polypeptides recognized by the prototype serum Wa in a dose-dependent manner (Fig. 3, lanes *3–11*); however, polypeptides of 190, 155, 138, 126, 44, and 23 kD, presumed components of RNAP I and III, were still immunoprecipitated. Identical results were obtained with two other sera that immunoprecipitated the RNAP II subunits,

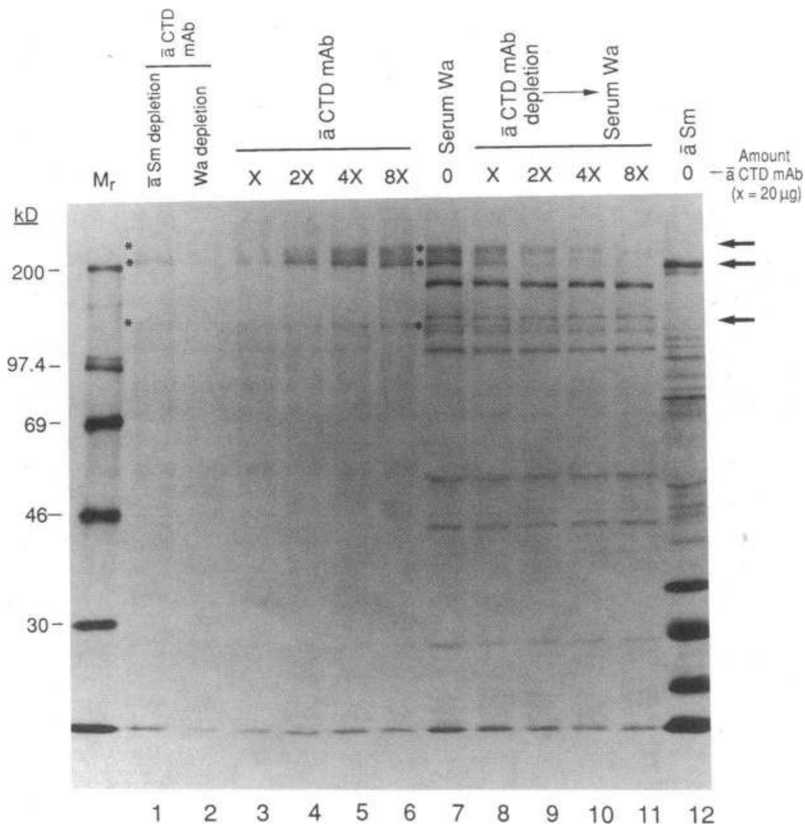


Figure 3. Depletion of RNAP II from [^{35}S]-methionine-labeled HeLa cell extracts by the anti-CTD mAb and human anti-RNAP II antibodies. The anti-CTD mAb immunoprecipitated the RNAP II large subunits (240-, 220-, and 145-kD polypeptides; denoted by asterisks and arrows) in a dose-dependent manner (lanes *3–6*), and effectively depleted these proteins from solution before immunoprecipitation with the prototype human serum Wa (lanes *7–11*). Immunoprecipitation studies shown in lanes *8–11* were performed with the serum Wa and the supernatants were derived from the immunoprecipitates shown in lanes *3–6*. A control immunoprecipitation performed with an anti-Sm serum from a patient with SLE is shown in lane *12*. The supernatant of the latter immunoprecipitation still contained RNAP II large subunits (240-, 220-, and 145-kD polypeptides; denoted by asterisks), which could be immunoprecipitated with the anti-CTD mAb (lane *1*), whereas the supernatant absorbed with serum Wa from lane *7* did not contain these polypeptides (lane *2*).

along with the putative polypeptides of RNAP I and III. In a reciprocal fashion, when HeLa cell extracts were absorbed with serum Wa and then probed with the anti-CTD mAb, the RNAP II subunits of 240, 220, and 145 kD were not immunoprecipitated (Fig. 3, lane *2*). In contrast, depletion of radiolabeled HeLa cell extracts using autoantibodies of different immunologic specificities (e.g., anti-Sm antibodies) did not affect specific antigen recognition by the anti-CTD mAb (Fig. 3, lane *1*). These findings indicate that the epitope bound by the monoclonal antibody to RNAP II resides on the same macromolecular structure recognized by the autoantibodies from sera of patients with systemic sclerosis.

Similar immunodepletion studies using human sera confirmed that the six smaller polypeptides (190, 155, 138, 126, 44, and 23 kD) immunoprecipitated by the anti–RNAP II sera were identical to those bound by the additional 54 sera from systemic sclerosis patients that did not bind RNAP II (data not shown).

Immunofluorescence studies. Indirect immunofluorescence was next used to identify the cellular location of the antigen(s) bound by the sera studied here. As shown by a representative example, all 13 systemic sclerosis sera that immunoprecipitated RNAP II subunits produced nucleoplasmic and nucleolar staining that was indistinguishable from that observed with a control antibody to RNAP I (Fig. 4, *A* and *B*, respectively). In contrast, staining produced with the anti-CTD mAb was limited to the nucleoplasm (Fig. 4, *C*). Therefore, to further define the site of binding of the human anti-RNAP II antibodies, double immunostaining was performed using the patient sera and the anti-CTD mAb. The location of the both antibodies was then determined with goat anti-human IgG conjugated to fluorescein isothiocyanate and goat anti-mouse IgG conjugated to Texas red. As shown by the orange fluorescence, the nucleoplasm was stained by both the anti-CTD mAb and anti–RNAP II sera (Fig. 4, *D*). In contrast, cells stained with normal human sera did not produce immunofluorescence, while those stained with anti-Sm antibodies (from human sera and the Y12 mAb) produced only finely speckled nuclear fluorescence (data not shown).

Inhibition of transcription from the adenovirus major late promoter and from the CMV immediate early promoter with IgG from systemic sclerosis sera. These studies were designed to test the ability of anti–RNAP II antibodies from patients to inhibit promoter-directed transcription in vitro. In these transcription assays, 10 μl of a Dignam extract was incubated for 30 min with IgG (from 50 μl of patient serum) bound to protein A-sepharose beads, before the addition of NTPs and the DNA template. The beads then were removed with centrifugation and the supernatant was used for the transcription from the DNA segments containing the adenovirus major late promoter and the CMV immediate early promoter. RNA was purified and analyzed on a 5% polyacrylamide/7 M urea gel (26). IgG from serum Wa and from a second serum that immunoprecipitated RNAP II subunits significantly inhibited RNAP II activity (Fig. 5 *A*, lane *2;* Fig. 5 *B*, lanes *3* and *4*). In contrast, normal human IgG (Fig. 5 *A* and *B*, lane *1*) and IgG from an anti-Sm patient serum did not produce any inhibition (Fig. 5 *B*, lane *2*). In this assay system, no transcription was detected when α-amanitin was used at a concentration of 1 μg/ml, which inhibits RNAP II activity, but not RNAP I activity (data not shown) (27).

Immunoreactivity of patient anti–RNAP II antibodies with the 220- and 145-kD polypeptides of RNA polymerase II. In immunoblots, 9 of 13 anti–RNAP II sera bound the 220-kD

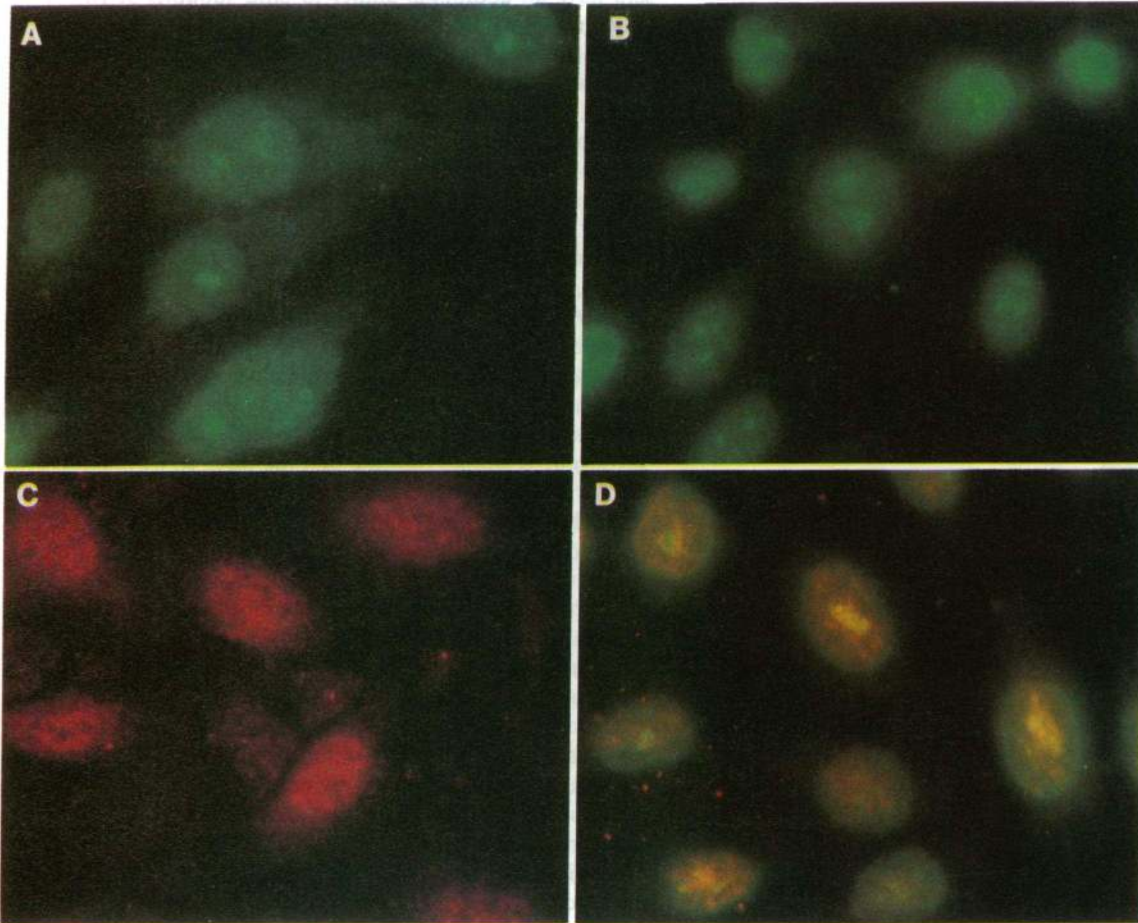


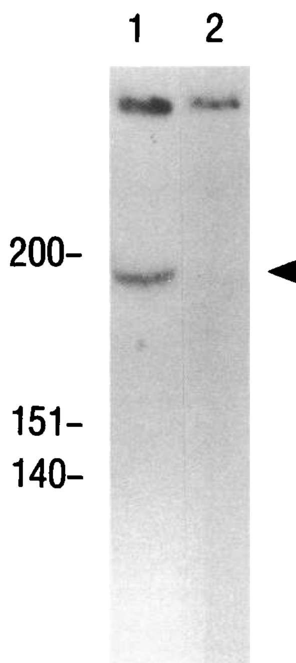
Figure 4. Immunofluorescence pattern of HEp-2 cells stained with serum Wa and the anti-CTD mAb. Serum Wa demonstrated nucleoplasmic and nucleolar staining (*A*). A serum which immunoprecipitated the apparent subunits of RNAP I and RNAP III, but did not recognize the large subunits of RNAP II, demonstrated the same pattern (*B*). Staining with the anti-CTD mAb was limited to the nucleoplasm (*C*). Double staining with serum Wa and the anti-CTD mAb showed nucleoplasmic staining (note the orange color produced by the colocalization of serum Wa (fluorescein isothiocyanate) and the anti-CTD mAb (Texas red) (*D*).

polypeptide and 11 of 13 recognized the 145-kD polypeptide of RNAP II (including three with weak reactivity), as demonstrated by representative examples (Fig. 6, lanes *2–5*). Only 1 of the 13 sera with antibodies to RNAP II as determined by immunoprecipitation did not bind either of the 220- or 145-kD subunits in immunoblots. Control anti-Sm, anti-La, anti-Ku, and normal human sera did not bind either polypeptide (Fig. 6, lanes *6–9*, respectively).

Discussion

These studies demonstrate the presence of antibodies to RNAP II through several different strategies. First, we found 13 sera, all from patients with systemic sclerosis, which were able to immunoprecipitate the 240-, 220-, and 145-kD polypeptide components of RNAP II. As demonstrated by an immunodepletion assay, these were the same polypeptides immunoprecipitated by the anti-RNAP II mAb 8WG16. In addition, the prototype serum Wa and a second human serum were able to inhibit RNAP II function in in vitro transcription assays. Finally, 12 of these 13 sera recognized either the 220- or 145-kD subunits in immunoblots, providing further evidence that these sera were not merely immunoprecipitating RNAP II via binding to subunits common to this polymerase and RNAP I and/or RNAP III.

It is noteworthy that the majority of the sera that immunoprecipitated RNAP II also bound the 220-kD polypeptide in immunoblots. Binding to a specific site of this protein can be inferred from the present studies. First, it is important to note that the RNAP II specific mAb 8WG16 (14, 15), as well as another RNAP II mAb (31), recognize the heptapeptide repeat of the CTD of the large 220-kD subunit, indicating that this region acts as an immunogenic epitope in animals. The 8WG16 mAb immunoprecipitates the 240-kD (IIO), 220-kD (IIA), and 145-kD (IIC) RNAP II subunits, but not the 180-kD (IIB) polypeptide derived from IIA by proteolysis, which deletes the CTD. This same pattern of immunoprecipitation was exhibited by the systemic sclerosis sera in our study suggesting that these sera recognized the CTD, but not the amino-terminal region corresponding to the 180-kD subunit. In addition, in preliminary studies we have recently expressed the CTD as a glutathione-*S*-transferase fusion protein, and have shown that representative anti–RNAP II patient sera recognized this fusion protein in immunoblots (32). Thus, a portion of the autoantigenic epitopes may reside on the heptapeptide repeats within the CTD. It has become clear that this domain



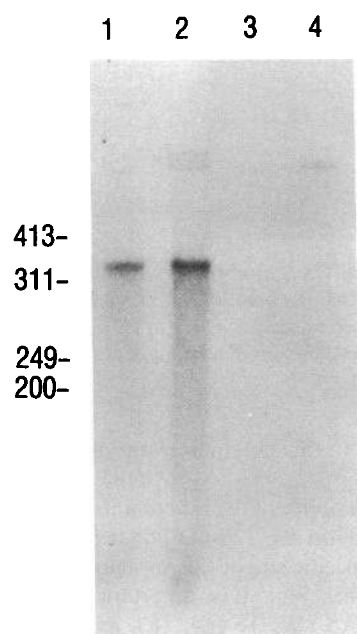


Figure 5. (*A*) Inhibition of transcription from the adenovirus major late promoter with IgG from a normal control serum (lane *1*) and serum Wa (lane *2*). (*B*) Inhibition of transcription from CMV immediate early promoter DNA with IgG from a normal control serum (lane *1*), an anti-Sm patient serum (lane *2*), and two anti-RNA polymerase II sera (lanes *3* and *4*). Assays for transcription were carried out as described under the Methods section and reaction products were resolved in a 5% acrylamide/7 M urea gel.

has an essential role in transcription, since it is a prerequisite for normal responses to activation signals at some promoters (2). Autoantibodies directed against this region, which is essential for cell viability, might be related to the pathogenesis of systemic sclerosis. However, precise epitope mapping analyses using molecular cloning and immunological approaches are needed to help answer this question.

The present immunofluorescence studies demonstrated that anti-RNAP II antibodies stain the same region within the nucleoplasm as the anti-CTD mAb, while the former antibodies also stained an antigen in the nucleolus. This observation can be rationalized by postulating that both the anti-CTD mAb and the patient antisera bind RNAP II, which is known to reside in the nucleoplasm (33), while the systemic sclerosis sera have an additional ability to bind subunits of RNAP I, which reside within the nucleolus.

12 of the 13 anti-RNAP II sera immunoprecipitated six additional, smaller polypeptides of 190, 155, 138, 126, 44, and 23 kD. Another 54 sera from patients with systemic sclerosis immunoprecipitated these same six polypeptides; immunodepletion studies confirmed that they were identical to the additional six polypeptides bound by the sera containing anti-RNAP II antibodies. One serum with anti-RNAP II antibodies also immunoprecipitated polypeptides of 155, 138, and 23 kD (Fig. 1, lane 9). RNAP I and RNAP III have a similar structure to RNAP II, each being composed of two large subunits (polypeptides of 190 and 126 kD, and 155 and 138 kD, respectively) and a set of smaller polypeptides, including those of 44 and 23 kD that are common to the three polymerases in the eukaryotes that have been studied (1). Indeed, immunological and phylogenetic studies indicate that the three RNA polymerases are derived from a common ancestral multimeric protein (1, 34). Thus, it appears that one of the 13 sera with anti-RNAP II antibodies immunoprecipitated RNAP III, while 12 of the 13 sera with these antibodies also apparently immunoprecipitated RNAP I and III. The additional 54 sera apparently contained antibodies to RNAP I and RNAP III but not to RNAP II.

Immunoprecipitation of RNAP II along with the other RNA polymerases could be secondary to the simultaneous presence in sera of antibodies that target a subunit(s) unique to each particle, or caused by antibodies that bind a protein common to these polymerases. Evidence in favor of the former supposition included the observation that the anti-RNAP II sera presumably were able to specifically bind RNAP I subunits since they stained the nucleolus in indirect immunofluorescence. Additionally, two of us (Y. Okano and T. Medsger) have recently found that representative samples of the sera that immunoprecipitated the apparent components of RNAP III, in addition to immunoprecipitation of the other polymerases, inhibited RNAP III activity in an in vitro transcription assay, and bound its unique subunits in immunoblots, indicating that these sera contain antibodies to the latter polymerase (35). On the other hand, we have now found that 12 of 13 of our anti-RNAP II sera immunoprecipitate the 23-kD subunit of RNAP II translated in vitro (Hirakata, M. unpublished observation); this subunit also is associated with RNAP I and RNAP III. In total, these results suggest that sera directed against RNA polymerases may contain antibodies that bind subunits specific for each polymerase, as well as antibodies that target shared subunits. Based on these observations, a hierarchy of autoantibody production to RNA polymerases may exist. An initial response may be directed against RNA polymerases I and/or III, followed by propagation to include a response against RNA polymerase II; alternatively, an initial response may be directed against smaller, shared subunits, with subsequent propagation to the larger, unique subunits of one or more of the polymerases.

The observation that these sera recognize more than one RNA polymerase is analogous to the finding that individual sera from patients with SLE often contain antibodies that target both proteins common to the U small nuclear ribonucleoproteins (U snRNPs) involved in premessenger RNA splicing, as well as antibodies that bind proteins unique to the individual snRNPs (36). It may be important for understanding the mechanism of autoantibody production in rheumatic diseases that autoantibodies often occur against multimeric enzymes that

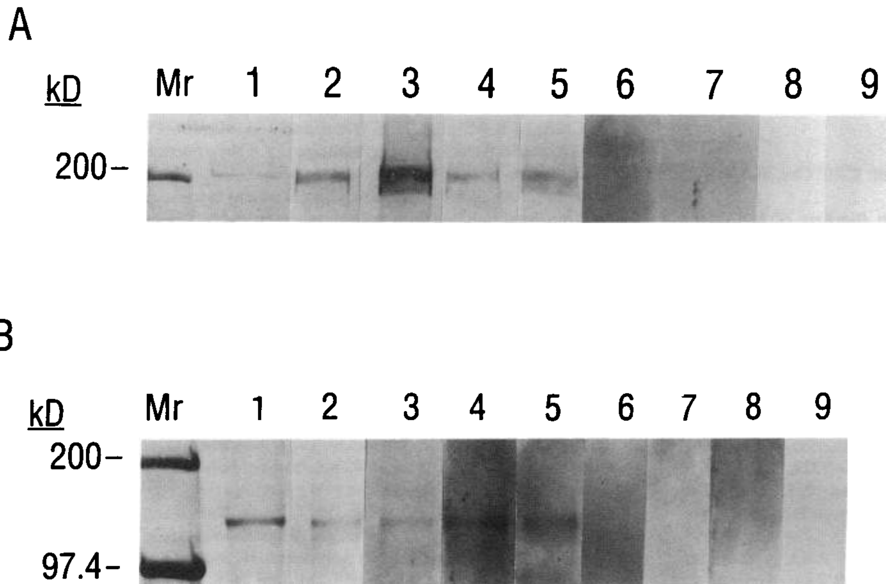


Figure 6. Immunoblot with human anti-RNAP II sera and control sera, using the 220-kD (*A*) and 145-kD (*B*) polypeptides of RNAP II as substrates. Bound antibodies were detected with alkaline phosphatase-conjugated anti-human IgG antibodies. Nitrocellulose strips after transfer were probed with anti-RNAP II sera (lanes *2–5*), an anti-Sm serum (lane *6*), an anti-SS B/La serum (lane *7*), an anti-Ku serum (lane *8*), and a normal serum (lane *9*), or stained with amido black (lane *1*).

share common components and that participate in a common function. To provide further insight into this point, it will be necessary to examine the immunoreactivity of autoantibodies toward each subunit of the RNA polymerases.

In the present study, we sought autoantibodies to RNA polymerases using immunoprecipitation assays, and confirmed the presence of antibodies to RNAP II using immunoblots of individual subunits and inhibition of transcription. In initial studies, we also carried out Ouchterlony double immunodiffusion assays using calf thymus extracts and HeLa cell extracts as antigens, and found that anti-RNA polymerase sera did not produce unidentified precipitin lines. The sera with anti-RNAP II antibodies also had only weak reactivities in immunoblots using crude HeLa extracts as substrates. Thus, to reliably identify antibodies to the subunits of RNAP II, it may be necessary to purify these components (or use recombinant proteins) for analyses in immunoblots or in other solid-phase assays.

In systemic sclerosis, specific autoantibodies are associated with disease subsets (37, 38). For example, anti-topoisomerase I, anti-RNAP I, and anti-U3 RNP (fibrillarin) antibodies are specific for systemic sclerosis with diffuse cutaneous involvement (37–39), while anticentromere and anti-Th RNP antibodies are typically found in patients with limited skin involvement (37, 40). In the present study, anti-RNAP II antibodies were detected in 13 (5%) sera with systemic sclerosis, of whom 10 had diffuse skin involvement. These antibodies were not found in sera from patients with closely related connective tissue diseases, including SLE, PM/DM, MCTD, and normal control sera. Thus, our findings suggest that anti-RNAP II antibodies may be a marker for a clinical subset of patients with systemic sclerosis with diffuse skin disease, analogous to the clinical associations of anti-topoisomerase I, anti-RNAP I, and anti-U3 RNP antibodies (38).

Regarding the clinical associations of autoantibodies in systemic sclerosis, we recently have found that anti-RNAP III occurred as frequently as antibodies to topoisomerase I and kinetochores in sera of these patients, and was a marker for diffuse cutaneous involvement and increased risk of renal disease (35). Taken together with the clinical association of antibodies to RNAP II, and the previously described association of anti-RNAP I with diffuse disease, these findings suggest that antibodies to the RNA polymerases may be specific for a subset of patients with systemic sclerosis with diffuse cutaneous involvement. Insights into why the transcription machinery is targeted in patients with this disease could provide important clues to its etiology. However, we would emphasize that further studies are needed to clarify the precise clinical significance of anti-RNAP II antibodies. Finally, we should also note that using a solid-phase radioimmunoassay, Stetler et al. found anti-RNAP I antibodies in the majority of sera from patients with SLE, MCTD, Sjögren's syndrome, and rheumatoid arthritis, in addition to patients with systemic sclerosis (17, 41). Differences in techniques may explain the discrepancies with our results, since these investigators used a solid-phase radioimmunoassay to detect antibodies to RNAP I, with inhibition of transcription and immunoblots performed with certain sera. However, we believe that immunoprecipitation of [^{35}S]-methionine labeled cell extracts is sensitive (although we do not know if it is as sensitive as radioimmunoassays), and that proteins of the RNA polymerases were not obscured by the presence of other bands in immunoprecipitates.

RNAP II is responsible for the synthesis of mRNA precursors in eukaryotes, and autoantibodies to this enzyme will be useful probes for further analysis of its structure and function. Studies to understand why autoantibodies arise against such a highly conserved enzyme could provide important insights into the pathogenic mechanisms of systemic sclerosis and related diseases.

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