Autoantibodies to RNA Polymerase II Are Common in Systemic Lupus Erythematosus and Overlap Syndrome

Specific Recognition of the Phosphorylated (IIO) Form by a Subset of Human Sera

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Abstract

Autoantibodies to RNA polymerases (RNAP) I, II, and III are reported to be highly specific for the diagnosis of scleroderma (systemic sclerosis, SSc). In the present study, the specificity of autoantibodies to RNAP I and III for SSc was confirmed by immunoprecipitation of ³⁵S-labeled proteins. However, we report here the previously unrecognized production of anti-RNAP II autoantibodies by 9–14% of patients with SLE and mixed connective tissue disease/overlap syndrome. 12 out of 32 anti-RNAP II positive sera (group 1) immunoprecipitated a diffuse 220–240-kD band identified as the largest subunit of RNAP II whereas the remaining 20 (group 2) immunoprecipitated preferentially the 240-kD phosphorylated (IIO) form of the large subunit. After pulse labeling, group 1 sera immunoprecipitated only the 220-kD (IIa) RNAP II subunit, whereas the diffuse IIa/IIO band plus the 145-kD second largest RNAP II subunit (IIc) were immunoprecipitated after several hours of cold chase, suggesting that these sera recognized primarily the largest subunit of RNAP II. Group 2 sera recognized the IIc subunit after pulse labeling, and immunoprecipitated the IIc and IIO, but not the IIa, subunits after cold chase. Although it has been suggested that autoantibodies to RNAP II are usually accompanied by anti-RNAP I/III in SSc, all but one of the anti-RNAP II positive sera from SLE or mixed connective tissue disease/overlap syndrome patients, as well as most of the SSc sera, were negative for anti-RNAP I/III. Moreover, in contrast to previous reports suggesting that anti-RNAP antibodies rarely coexist with other SSc subset marker antibodies, anti-RNAP II antibodies were often accompanied by anti-Ku, anti-nRNP, or anti-topoisomerase I autoantibodies in the present study. We conclude that autoantibodies to RNAP II are not a specific marker for SSc, whereas autoantibodies to RNAP I/III are associated primarily with SSc. In addition, we have identified two distinctive patterns of RNAP II antigen recognition by autoanti-

bodies, one of them characterized by specific recognition of the transcriptionally active (phosphorylated) form of RNAP II. The clinical significance of these different patterns remains to be determined. (*J. Clin. Invest.* 1994. 94:1981–1989.) Key words: RNA polymerase II • autoantibodies • systemic lupus erythematosus • scleroderma • overlap syndrome

Introduction

Certain autoantibodies are highly characteristic of specific diseases, including anti-Sm in SLE (1), anti-topoisomerase I in scleroderma (systemic sclerosis [SSc])¹ (2), and anti-aminoacyl-tRNA synthetases in polymyositis/dermatomyositis (PM/DM) (3, 4). Other autoantibodies are more closely associated with particular symptoms regardless of the diagnosis, such as anti-Ro/SSA and -La/SSB with sicca syndrome (5–7), and anti-centromere and anti-nRNP antibodies with Raynaud's phenomenon (8–10). These autoantibodies may be useful diagnostically as well as prognostically. Accurately defining the clinical characteristics of patients with a new autoantibody specificity is the first step to establishing its clinical significance. Recent studies have stressed the specificity of autoantibodies to RNA polymerases (RNAP) I, II, and III for the diagnosis of SSc (11–15). In the present studies, we confirmed the diagnostic specificity of autoantibodies to RNAP I/III. However, we now report a previously unrecognized association of autoantibodies to RNAP II with SLE and SLE-overlap syndrome. In this study, anti-RNAP II autoantibodies were detected in 9–14% of patients with SLE and mixed connective tissue disease (MCTD)/overlap syndrome as well as 20% of patients with SSc without overlapping features.

Methods

Diagnostic criteria. The clinical diagnoses of SLE, SSc, and RA were made based on the ACR criteria (16–18). Sjögren's syndrome was diagnosed using the criteria of the Ministry of Health and Welfare, Japanese government, and PM/DM were diagnosed by Bohan's criteria

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Received for publication 22 April 1994 and in revised form 5 July 1994.

J. Clin. Invest.

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0021-9738/94/11/1981/09 \$2.00

Volume 94, November 1994, 1981–1989

1. *Abbreviations used in this paper:* CTD, carboxyl-terminal domain; MCTD, mixed connective tissue disease; PM/DM polymyositis/dermatomyositis; RNAP, RNA polymerase; IIa, nonphosphorylated form of RNA polymerase II; IIO, phosphorylated form of RNA polymerase II; IIa, nonphosphorylated form (220 kD) of RNA polymerase II largest subunit; IIO, phosphorylated form (240 kD) of RNA polymerase II largest subunit; IIc, second largest subunit (145 kD) of RNA polymerase II.

(19, 20). Patients with two or more features each of SLE, SSc, and PM/DM were classified as MCTD/overlap syndrome. In analyzing the clinical characteristics of SLE patients with anti-RNAP II autoantibodies, patients meeting four or more SLE criteria along with overlapping features of SSc or PM/DM were categorized as SLE overlap syndrome.

Human sera and monoclonal antibodies (mAbs). Sera were obtained from patients with SLE, other autoimmune disorders, pulmonary tuberculosis, or cervical carcinoma followed at Keio University Hospital, National Murayama Hospital, or Fussa Hospital (Tokyo, Japan), or at the University of North Carolina Hospitals (Chapel Hill, NC). Additional serum samples were obtained from patients fulfilling ACR criteria for SLE seen at the Rockefeller University Hospital (New York, NY). Autoantibodies to nRNP, Sm, Ro/SS-A, La/SS-B, Jo-1, topoisomerase I, Ku, and Ki (SL) were determined by double immunodiffusion as described previously using appropriate reference sera (21). Specificities were also confirmed by ^{35}S -immunoprecipitation, with the exception of anti-La/SS-B and anti-Ki. Anti-Su was defined by immunoprecipitation of the characteristic 100/102-kD doublet and 200-kD polypeptides (Satoh, M., J. J. Langdon, C. H. Chou, D. McCauliffe, E. Treadwell, T. Ogasawara, M. Hirakata, A. Suwa, P. L. Cohen, R. A. Eisenberg, and W. H. Reeves. *Clin. Immunol. Immunopathol.* In press).

Reference human autoimmune serum specific for anti-RNAP I/III (12, 15), was provided by Dr. Yutaka Okano (University of Pittsburgh, Pittsburgh, PA). Reference human autoimmune sera specific for anti-RNAP II were described previously (12, 22). Murine mAb 8WG16, specific for the carboxyl-terminal domain (CTD) of the largest subunit of RNAP II (23, 24), was a gift of Dr. Nancy E. Thompson (McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI).

Immunoprecipitation. The proteins recognized by human autoimmune sera were determined by immunoprecipitation of radiolabeled cell extracts and 8% SDS-PAGE as described (25). Briefly, human K562 erythroleukemia cells were labeled for 14 h with $20\ \mu\text{Ci}/\text{ml}$ [^{35}S]methionine and cysteine (DuPont-NEN, Boston, MA) in methionine-free RPMI 1640 containing 10% PBS-dialyzed FBS and 3% regular RPMI. The cells were resuspended at $10^7/\text{ml}$ in ice cold 0.5 M NaCl NET buffer (500 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl, pH 7.5) containing 0.3% NP-40, 0.5 mM PMSF, 0.3 TIU/ml aprotinin, sonicated intermittently for 120 s, and cleared by centrifugation. Protein A-Sepharose CL4B beads (Pharmacia Fine Chemicals, Piscataway, NJ) were incubated for 1 h with $10\ \mu\text{l}$ of human serum or $3\ \mu\text{l}$ of mAb 8WG16 (1 mg/ml), and then washed twice with 0.5 M NaCl NET/NP-40 buffer. Cell extract from 2×10^6 cells was incubated with antibody-coated beads for 1 h at 4°C followed by washing three times with 0.5 M NaCl NET/NP-40, and twice with NET buffer. Radiolabeled proteins binding to the beads were analyzed by SDS-PAGE and autoradiography.

Alkaline phosphatase treatment of RNAP II. ^{35}S -labeled cell extract was immunoprecipitated with $10\ \mu\text{l}$ of human autoimmune sera or $3\ \mu\text{l}$ of mAb 8WG16 as above. The beads were washed three times with 0.5 M NaCl NET/NP-40, once with NET, and twice with 20 mM Tris-HCl, 150 mM NaCl, pH 8.0, 1 mM MgCl_2 . The beads were then resuspended in $50\ \mu\text{l}$ of 20 mM Tris-HCl, pH 9.6, 1 mM MgCl_2 , containing 2 units of calf intestine alkaline phosphatase (Boehringer Mannheim Corp., Indianapolis, IN), and incubated for 0.5 or 5 h at 37°C with intermittent mixing. The same treatment without alkaline phosphatase was used as a control. Digestion was stopped by adding $25\ \mu\text{l}$ of SDS sample buffer and the samples were analyzed by SDS-PAGE as above.

Pulse-chase analysis. K562 cells were pulse-labeled with [^{35}S]methionine and cysteine as described previously (26). Briefly, 1.2×10^8 cells were washed twice with PBS, preincubated for 30 min in 2 ml of methionine-free RPMI 1640 containing 10% PBS dialyzed FBS, and labeled for 15 min at 37°C by adding 1.5 mCi of [^{35}S]methionine and cysteine. After pulse labeling, 45 ml of cold RPMI 1640 was added and the cells were collected by centrifugation and washed with PBS. 2×10^7 pulse-labeled cells were harvested immediately, and the remaining cells were cultured at 37°C in five tissue culture flasks (2×10^7 cells in 5 ml of complete medium per flask). One flask (2×10^7 cells) was harvested after 1, 2, 4, 8, and 16 h of cold chase, respectively. The cells

were washed once with PBS and frozen. The cells were then thawed and sonicated at $2 \times 10^7/\text{ml}$ in 0.5 M NaCl NET/NP-40, and the extract was cleared by centrifugation and immunoprecipitated with $3\ \mu\text{l}$ mAb 8WG16, or $10\ \mu\text{l}$ of anti-RNAP II positive autoimmune sera as above. Beads were washed three times with 0.5 M NaCl NET/NP-40 buffer and once with NET buffer.

Clinical analysis. The prevalence of autoantibodies to RNA polymerase II and I/III was determined by immunoprecipitation for unselected rheumatic disease patients seen at Keio University Hospital, National Murayama Hospital, or Fussa Hospital, or at the University of North Carolina Hospitals. All patients were classified as described above using clinical criteria for SLE, MCTD/overlap syndrome, PM/DM, SSc, Sjögren's syndrome, or RA. Sera from healthy control subjects, patients with pulmonary tuberculosis, or patients with cervical carcinoma were also examined. The association of anti-RNAP II with autoantibodies to nRNP, Sm, Ro/SS-A, La/SS-B, Ku (p70/p80), Ki, Jo-1, and topoisomerase I (Scl-70) by double immunodiffusion and/or immunoprecipitation were also studied.

Results

Immunoprecipitation with human autoimmune sera. Eukaryotic RNAP II is a multisubunit complex consisting of 10–14 polypeptides ranging in size from 10–220 kD (27). The precise subunit composition of the enzyme remains somewhat controversial, and the structure and function of the individual subunits of human RNAP II are still poorly characterized (28, 29). Autoantibodies to RNAP II have been defined previously based on their ability to immunoprecipitate the two largest subunits of 220 kD and 145 kD, respectively, as well as the 240-kD phosphorylated form of the largest subunit (12, 14). Immunoprecipitation of the strongly radiolabeled 220–240- and 145-kD subunits is characteristic of murine mAbs as well as autoimmune sera specific for RNAP II. The smaller subunits of RNAP II are labeled inefficiently, and are not consistently visualized in immunoprecipitation.

We screened human autoimmune sera for anti-RNAP II autoantibodies by immunoprecipitation, comparing with the mobility by SDS-PAGE of the characteristic 220–240- and 145-kD polypeptides immunoprecipitated by the murine anti-RNAP II mAb 8WG16 (Fig. 1, lane 3). Immunoprecipitation of RNAP I/III components was confirmed using human reference sera previously described by Hirakata et al. (12) (lane 1) and Okano et al. (15), as well as anti-RNAP I, II, and III reference serum (12, 22) (lane 2). Fig. 1 shows the immunoprecipitation patterns of representative sera containing autoantibodies to RNAP II from eight patients with SLE (lanes 4–11) and two patients with SLE-overlap syndrome (lanes 12 and 13). All 10 sera immunoprecipitated 220–240- and 145-kD polypeptides comigrating exactly with the largest and second largest subunits of RNAP II that were immunoprecipitated by mAb 8WG16 (lane 3). Like 8WG16, many sera immunoprecipitated a diffuse band or bands in the 220–240-kD range (Fig. 1, lanes 4–8). This pattern has been shown previously to reflect phosphorylation of the CTD of the 220-kD subunit RNAP II, resulting in a mobility shift to 220–240 kD. The nonphosphorylated and phosphorylated forms of RNAP II have been designated IIA and IIO, respectively, and the nonphosphorylated and phosphorylated forms of the largest subunit have been termed Ila and Ilo, respectively (12, 30, 31). Some sera immunoprecipitated the 240-kD (Ilo), but not the 220-kD (IIa) form along with the 145-kD (Iic) polypeptide (Fig. 1, lanes 9–13). With one exception (lane 4), all anti-RNAP II positive sera from patients

13), anti-Ro/SS-A (lanes 6, 8, and 9), and anti-Sm/RNP (U1-A; lanes 4, 5, 8, 11–13) in some sera. Molecular weight standards in kD are shown on the right. I, two largest subunits of RNA polymerase I; III, two largest subunits of RNA polymerase III.

with SLE and SLE-overlap syndrome failed to immunoprecipitate RNAP I/III components, as indicated by comparison with human anti-RNAP I/III reference serum (lane 1). The only exception was one serum from a patient with SLE (lane 4) that immunoprecipitated the two largest subunits of RNAP I (197 and 126 kD) strongly as well as the two largest subunits of RNAP II. The two largest subunits of RNAP III (155 and 138 kD) were also seen weakly. Normal human serum did not immunoprecipitate components of either RNAP II or RNAP I/III (lane 14).

Alkaline phosphatase treatment of RNAP II. To confirm that the diffuse band migrating at 220–240 kD and the 240-kD band immunoprecipitated by some sera, represented the large subunit of RNAP II and its phosphorylated forms, immunoprecipitates were treated with alkaline phosphatase (32). The mobility of the 220–240-kD band was unaffected even after 5 h incubation at 37°C if alkaline phosphatase was not added (Fig. 2 A). As shown in Fig. 2 B, there was no clear difference in this pattern

after 30 min of alkaline phosphatase digestion, although the intensity of the 240-kD band in lanes 4–6 may have been somewhat weaker. However, the mobility was reduced significantly after 5 h of alkaline phosphatase treatment (Fig. 2 C). In lane 1, although the 240-kD band immunoprecipitated by anti-RNAP I, II, III serum remained visible, the diffuse 220–240-kD band shifted to 220 kD. The diffuse 220–240 kD band immunoprecipitated by mAb 8WG16 (lane 2) as well as by human autoimmune sera (lanes 3 and 4) was also shifted to ~220 kD, as shown previously using biochemically purified RNAP II (32). The 240-kD band in lanes 5 and 6 shifted to 220–230 kD after dephosphorylation. These results are consistent with nearly complete (lanes 2 and 4) or partial (lanes 3, 5, and 6) dephosphorylation of the affinity-purified 240-kD band, and provide additional evidence that the diffuse 220–240-kD band immunoprecipitated by the patient's sera consists of the unphosphorylated plus phosphorylated forms of the largest subunit of RNAP II (30). In addition, the data suggest that some sera preferentially

Figure 2. Alkaline phosphatase treatment of RNAP II. Radiolabeled K562 cell extract was immunoprecipitated with anti-RNAP I, II, III reference sera (lane 1), mAb 8WG16 (lane 2), or human autoimmune sera that immunoprecipitate either the diffuse 220–240-kD band (lanes 3 and 4) or the 240-kD form of the large RNAP II subunit (lanes 5 and 6). The beads were incubated in buffer at 37°C for 5 h without alkaline phosphatase (A), with alkaline phosphatase for 30 min (B), or with alkaline phosphatase for 5 h (C). The mobility of the 220–240-kD band was unaffected if alkaline phosphatase was not added (A). Differences were minimal after 30 min of alkaline phosphatase digestion, but significant mobility differences were apparent after 5 h of alkaline phosphatase treatment (C). The mobilities of the 145-kD RNAP II subunit and the 70- and 80-kD Ku proteins were unaffected by alkaline phosphatase treatment, even after 5 h. Molecular weight standards are indicated on the right.

line phosphatase for 30 min (B), or with alkaline phosphatase for 5 h (C). The mobility of the 220–240-kD band was unaffected if alkaline phosphatase was not added (A). Differences were minimal after 30 min of alkaline phosphatase digestion, but significant mobility differences were apparent after 5 h of alkaline phosphatase treatment (C). The mobilities of the 145-kD RNAP II subunit and the 70- and 80-kD Ku proteins were unaffected by alkaline phosphatase treatment, even after 5 h. Molecular weight standards are indicated on the right.

Figure 3. Pulse-chase analysis. K562 cells were pulse labeled with [35 S]methionine and cysteine for 15 min at 37°C followed by 1, 2, 4, 8, and 16 h of cold chase, respectively. Cell extracts were immunoprecipitated with mAb 8WG16 (A), anti-RNAP I, II, III-positive serum (B), or anti-RNAP II-positive autoimmune sera (C and D). Note that mAb 8WG16, which is specific for the 220-kD RNAP II subunit, and certain autoimmune sera (B and C) immunoprecipitated mainly that subunit after 15 min of pulse labeling (A), but with increasing cold chase, the mobility of the 220-kD subunit was altered to the characteristic 220–240-kD pattern (arrows) and progressively more of the 145-kD polypeptide was immunoprecipitated (A, arrow). Other sera immunoprecipitated the 145-kD subunit after pulse labeling, with immunoprecipitation of the 240-kD band after cold chase (D). Positions of the two largest subunits of RNAP I (Ia and Ib, 197 and 126 kD, respectively) and III (IIIa and IIIb, 155 and 138 kD respectively) are indicated (B). Molecular weight standards are shown on the right. Immunoprecipitation of extracts from cells labeled for 14 h using anti-RNAP I, II, III human serum (I-III) or 8WG16 (II) are shown on the left of each panel.

immunoprecipitate the phosphorylated (IIO) form of RNAP II. The slight differences in the extent of the mobility shift seen after purification of RNAP II by different autoimmune sera may be related to differences in the specificity of the autoantibodies. Since alkaline phosphatase treatment was performed on the beads, certain sera might contain autoantibodies that limit the accessibility of the enzyme to one or more of the phosphorylated sites of the RNAP II large subunit.

Pulse chase analysis. RNAP II has been shown previously to be a multiprotein complex consisting of at least 10 subunits ranging in size from 10 to 220 kD (27, 30, 33). Although the experiments presented in Figs. 1 and 2 strongly suggested that certain sera from patients with SLE or overlap syndrome immunoprecipitate RNAP II, the specificity of the autoantibodies could not be determined because sera with antibodies to any of the subunits of RNAP II would be expected to coimmunoprecipitate the other subunits. Pulse-chase experiments were performed to address the question of autoantibody specificity (Fig. 3). As expected, mAb 8WG16, which is specific for the largest RNAP II subunit, immunoprecipitated mainly that subunit after

15 min of pulse labeling (Fig. 3 A). With increasing cold chase, the mobility of the 220-kD subunit was altered to the characteristic 220–240-kD pattern, consistent with posttranslational modification (phosphorylation) of that subunit (30, 31). In addition, 8WG16 immunoprecipitated progressively more of the 145-kD polypeptide with increasing time of cold chase (Fig. 3 A, arrow), consistent with assembly of the 220–240- and 145-kD subunits into a particle. Indeed, it has been suggested previously that the largest and second largest subunits of RNAP II interact directly with one another, probably at multiple sites (27). Immunoprecipitation of the remaining subunits was visualized less clearly, consistent with previous observations (12). Immunoprecipitation using many of the anti-RNAP II sera from patients with SLE or overlap syndrome displayed a pattern nearly identical to that of mAb 8WG16 in pulse-chase experiments (Fig. 3, B and C). In the case of a serum containing autoantibodies to RNAP I and III, as well as II (Fig. 3 B), immunoprecipitation of the components of RNAP II displayed a pattern similar to that of 8WG16, whereas the components of RNAP I also displayed a pattern compatible with autoantibody

Table I. Prevalence of Autoantibodies to RNA Polymerases by Immunoprecipitation in Japanese, Caucasian, and Black Patients with Systemic Rheumatic Diseases

Diagnosis	Japanese			Caucasian			Black		
	n	RNAPII	I/III	n	RNAPII	I/III	n	RNAPII	I/III
SLE	76	9%	0	29	7%	0	33	18%	3%
MCTD/Overlap	42	14%	0	1	0	0	3	67%	0
PM/DM	26	0	0	2	0	0	ND		
Scleroderma	35	20%	3%	9	11%	22%	3	33%	33%
Sjogren's	13	0	0	ND			ND		
RA	35	0	0	6	0	0	4	0	0
Control	32	0	0	16	0	0	6	0	0

Control; Japanese: 7 healthy, 25 patients with pulmonary tuberculosis; Caucasian: 9 healthy, 7 patients with cervical carcinoma; Black: 6 patients with cervical carcinoma. ND, not determined.

recognition of the largest subunit, i.e., immunoprecipitation of mainly the large (197 kD) subunit after pulse labeling, followed by coimmunoprecipitation of progressively more of the 126-kD (second largest) subunit during cold chase (Fig. 3 B; Ia and Ib, respectively). In contrast, the two largest subunits of RNAP III were immunoprecipitated with comparable intensity throughout the pulse-chase experiment (Fig. 3 B; IIIa and IIIb, respectively), consistent with the presence of autoantibodies that recognize both RNAP III subunits or else the rapid assembly of these subunits into a particle. Sera that immunoprecipitated primarily the 240-kD form of the large RNAP II subunit displayed a very different pattern (Fig. 3 D). These sera immunoprecipitated exclusively the 145-kD subunit after pulse labeling and after short periods of cold chase, but immunoprecipitated the 145-kD polypeptide as well as the 240-kD, but not the 220-kD, form of the large subunit after 2–16 h of cold chase. The time course of immunoprecipitation of the 240-kD band closely paralleled that of posttranslational modification of the 220-kD polypeptide.

Prevalence of autoantibodies to RNAP II in systemic rheumatic disease. Screening of randomly selected Japanese ($n = 227$), Caucasian ($n = 47$), and Black ($n = 43$) rheumatic disease patients revealed that anti-RNAP II autoantibodies were not restricted to SSc, but were also detected at relatively high frequency in SLE and MCTD/overlap syndrome (Table I). Sera from 7 of 76 Japanese (9%), 2 of 29 Caucasian (7%), and 6 of 33 Black (18%) SLE patients without overlapping features of SSc immunoprecipitated RNAP II. In overlap syndromes, anti-RNAP II antibodies were detected in 6 of 42 Japanese patients (14%), and in 2 of 3 Black patients (67%) in this category. Autoantibodies to RNAP II were frequent in SSc (7 of 35 Japanese (20%) and 2 of 12 Caucasian and Black Americans (17%) with this diagnosis). In contrast, anti-RNAP II antibodies were not detected in PM/DM, Sjögren's syndrome or RA, or controls without systemic rheumatic disease, including patients with pulmonary tuberculosis and cervical carcinoma, and healthy individuals. In contrast to the association of anti-RNAP II antibodies with several clinical subsets, anti-RNAP I/III were highly specific for SSc. Autoantibodies to RNAP I/III were present in only one serum from a Black SLE patient out of a total of 138 SLE patients examined (Fig. 1, lane 4, and Table I). Autoantibodies to RNAP I/III were detected almost exclusively in patients with SSc in all racial groups.

Clinical features of anti-RNAP II-positive SLE patients.

The relatively high prevalence of autoantibodies to RNAP II in sera from patients with SLE or overlap syndromes contrasts sharply with previous observations (12). For that reason, the clinical presentations of patients with SLE or SLE overlap syndrome were examined closely for features of SLE and SSc. All 19 patients met four or more ACR criteria for the classification of SLE. Detailed clinical information was available for 17 patients (Table II). In addition to manifestations of relatively low diagnostic specificity such as arthritis and antinuclear antibodies, most patients had characteristic clinical symptoms and immunological abnormalities of SLE such as malar rash, nephritis, serositis, anti-DNA antibodies, and anti-Sm antibodies. Features of SSc were common in the four patients with SLE overlap syndrome (patients 6–8 and 17), but were unusual in patients carrying the diagnosis of SLE without overlapping features of SSc and/or PM/DM (patients 1–5 and 9–16). Of the latter group, 5 of 13 patients had Raynaud's phenomenon, and one had pulmonary fibrosis. None of these patients had proximal scleroderma, sclerodactyly, or digital pitting scars. Autoantibodies to Sm were detected in 5 of the 17 sera, and anti-dsDNA antibodies were present in 14 of 17. Autoantibodies to topoisomerase I (Scl-70) were detected in one patient with SLE-SSc overlap syndrome and one patient with SLE (Table II, cases 6 and 10, respectively).

Among these patients, 9 of 17 with anti-RNAP II had Raynaud's phenomenon; however, 7 of the 9 also had anti-nRNP antibodies, which have been reported previously to be associated with Raynaud's phenomenon (10, 34). Among the eight anti-RNAP II-positive patients whose sera did not contain anti-nRNP antibodies, only two had Raynaud's phenomenon, suggesting that anti-nRNP rather than anti-RNAP II antibodies may be associated with Raynaud's phenomenon in these patients. Thus, the clinical manifestations exhibited by these patients suggested that autoantibodies to RNAP II in SLE patients were not limited to those patients with overlapping features of SSc. These observations further support the idea that anti-RNAP II antibodies are not specific for SSc.

Immunoprecipitation of the phosphorylated and unphosphorylated forms of the largest subunit of RNAP II. 12 out of 32 anti-RNAP II-positive sera immunoprecipitated the diffuse 220–240-kD band consisting of the phosphorylated (IIo) and unphosphorylated (IIa) forms of the largest subunit (group 1, Table III). The remaining 20 patients' sera immunoprecipitated only the IIo form (group 2, Table III). All six sera from Japanese

Table II. Clinical Features of Patients with SLE and SLE Overlap Syndrome with Anti-RNA Polymerase II

Case	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Age	24	27	31	32	46	33	34	45	38	40	22	38	38	43	47	66	45
Race	J	J	J	J	J	J	J	J	W	W	B	B	B	B	B	B	B
SLE																	
1. Malar rash	+	+	-	-	-	+	+	-	-	-	-	+	-	+	+	-	-
2. Discoid	-	-	+	-	-	-	-	-	-	-	-	-	+	+	+	-	-
3. Photosensitivity	+	-	-	-	-	+	-	+	-	+	-	-	-	+	+	-	-
4. Oral ulcers	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-
5. Arthritis	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6. Serositis	-	-	-	-	-	-	+	+	+	-	+	-	+	+	-	+	-
7. Renal disorder	-	+	+	+	+	-	+	-	+	+	+	+	-	-	-	-	+
8. Neurologic	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
9. Hematologic	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+
10. Immunologic	LD	DS	LD	LB	D	L	LD	LD	D	D	D	DS	D	D	D	D	S
		B	B				S	S									
11. ANA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PM/DM	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+
Scleroderma																	
Proximal	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Sclerodactyly	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-
Pitting scars	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Pulmonary fibrosis	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+
Raynaud's	-	+	+	-	+	+	+	+	+	-	-	-	-	-	+	-	+
Autoantibodies																	
RNA polymerase II	1	2	1	2	2	2	2	2	1	2	1	2	2	2	1	2	1
nRNP	-	+	-	-	+	+	+	+	-	-	+	+	-	-	+	-	+
Sm	-	+	-	-	-	-	+	+	-	-	-	+	-	-	-	-	+
Ro	+	-	-	+	+	-	+	-	-	-	+	+	+	-	-	+	+
La	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-
Ku	-	-	-	+	-	-	-	+	-	-	-	+	+	-	+	-	+
Su	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	+
Others						Tp				*1					*2		

Cases 1-5, 9-16: SLE; Cases 6-8, 17: SLE-overlap syndrome. Cases 1-16: female; Case 17: male. *Race*: J, Japanese; W, White (Caucasian); B, Black (African-American). *Immunologic abnormalities*: L, LE cell; D, anti-dsDNA antibodies; B, biological false positive result for serological test for syphilis. S, anti-Sm antibodies; ANA, antinuclear antibodies. *Autoantibodies*: For RNA polymerase II, 1 denotes group 1, unphosphorylated (IIa) and phosphorylated (IIo) form, while 2 denotes group 2, phosphorylated (IIo) form only. Tp, anti-topoisomerase I antibodies; *1, Antibodies to ribosomal P and topoisomerase I; *2, Antibodies to RNA polymerase I and III.

SSc patients that contained anti-RNAP II without anti-RNAP I/III immunoprecipitated the phosphorylated form of RNAP II only. In contrast, all three sera from SSc patients in group 1 also contained anti-RNAP I/III.

Table III. Prevalence of Phosphorylated and Unphosphorylated Form of the Largest Subunit of RNA Polymerase II Immunoprecipitated by Autoimmune Sera

	Japanese		Caucasian		Black	
	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
SLE	3	4	1	1	2	4
Overlap	2	4	0	0	1	2
Scleroderma	1	6	1	0	1	0

Group 1: phosphorylated (IIo) and unphosphorylated (IIa) form; Group 2: phosphorylated (IIo) form only. None of the autoimmune sera immunoprecipitated only unphosphorylated form.

Association of anti-RNAP II with other autoantibodies. Among the 19 anti-RNAP II-positive SLE patients, anti-nRNP antibodies were found in 10 cases (Table IV). Anti-Ro antibodies were found in 10, and anti-Sm and anti-Su in 6 cases each. However, these antibodies are relatively common in SLE patients, and they were not detected significantly more frequently in the anti-RNAP II-positive patients than in anti-RNAP II negative individuals (data not shown). Autoantibodies to Ku were noted in 2 out of 10 Japanese patients and 4 out of 9 American patients with SLE and anti-RNAP II antibodies. Anti-topo I antibodies were found in 2 of 19 patients with SLE or SLE-overlap syndrome. These results argue against the idea that autoantibodies to RNAP II and topoisomerase I or other SSc subset marker autoantibodies are usually mutually exclusive (14, 35).

Discussion

Previous studies have suggested that autoantibodies to RNA polymerases, including RNAP II, serve as diagnostic markers

Table IV. Prevalence of Other Autoantibodies in Patients with Anti-RNA Polymerase II Antibodies

Autoantibodies	SLE (15)	SLE-OL (4)
nRNP	6/15	4/4
Sm	3/15	3/4
Ro	8/15	2/4
La	4/15	0/4
Ku	4/15	2/4
Su	5/15	1/4
Ribosomal P	2/15	0/4
Topoisomerase I	1/15	1/4

SLE: 7 Japanese, 2 Caucasian, and 6 Black patients. SLE-OL, SLE overlap syndrome: 1 Black and 3 Japanese patients.

for scleroderma (11–15). Although the present studies are in agreement with previous reports regarding the diagnostic specificity of autoantibodies to RNAP I/III, anti-RNAP II antibodies appear to be less specific for scleroderma than they were believed to be previously. The identification of human autoantibodies to RNAP II in the present study was based on immunoprecipitation of characteristic polypeptides of 220–240 and 145 kD comigrating with proteins immunoprecipitated by mAb 8WG16 and human reference sera, pulse-chase analysis, and the decreased mobility of the Ilo (phosphorylated) form of the large subunit immunoprecipitated by the human autoimmune sera and the reference murine mAb after alkaline phosphatase treatment. In addition, both the 220- and the 240-kD proteins immunoprecipitated by SLE sera could be absorbed by preincubating the extract with anti-RNAP II reference sera (data not shown). Based on all of these criteria, the 220–240 and 145-kD polypeptides immunoprecipitated by the human autoimmune sera in the present study were considered to be subunits of RNAP II. Although we could not completely exclude the possibility that some of the sera immunoprecipitating RNAP II contained autoantibodies to other proteins bound to RNAP II (32, 36), no other common proteins were seen on immunoprecipitation. In addition, it is possible that autoantibodies to RNAP II in some SLE sera actually are directed against another antigen that carries antigenic determinants crossreactive with RNAP II. Regarding this point, three out of four sera originally selected for the reactivity with the G8 antigen, a novel autoantigen having sequence similarity to the CTD of RNAP II (37), immunoprecipitated RNAP II (M. Satoh and W. H. Reeves, unpublished observations [these patients are not included in the present study]). The finding that SLE sera display two different RNAP II recognition patterns may argue against crossreactivity of RNAP II with another antigen, although further studies are necessary.

Autoantibodies specific for the phosphorylated form of the RNAP II largest subunit. All of the patients' sera with anti-RNAP II described previously immunoprecipitated both the phosphorylated (Ilo) and unphosphorylated (Ila) forms of the large subunit. In the present study, more than half of the human anti-RNAP II autoimmune sera preferentially immunoprecipitated the phosphorylated (Ilo) form of the largest subunit without the Ila form (Fig. 1). Identity of this band with the phosphorylated form of the large subunit was confirmed by its shift in mobility and reduced intensity after alkaline phosphatase

Figure 4. Interpretation of pulse chase experiments. Three possible explanations were found for the immunoprecipitation by some sera of only the 145-kD (Ilo) subunit after pulse labeling, with immunoprecipitation of the Ilo and 240-kD (Ilo) subunits, but not the 220-kD (Ila) subunit, after cold chase (see Fig. 3 D). (A) Autoantibody recognition of an epitope of the Ilo (145 kD) subunit that is exposed when bound to the Ilo (240 kD) subunit, but covered when bound to the Ila (220 kD) subunit. (B) Autoantibody recognition of an epitope specific to the (labeled or unlabeled) Ilo subunit. (C) Autoantibody recognition of an epitope composed of both the (labeled or unlabeled) Ilo and Ila subunits.

unit, but covered when bound to the Ila (220 kD) subunit. (B) Autoantibody recognition of an epitope specific to the (labeled or unlabeled) Ilo subunit. (C) Autoantibody recognition of an epitope composed of both the (labeled or unlabeled) Ilo and Ila subunits.

digestion (Fig. 2) and the fact that the 240-kD band could be absorbed completely with reference autoimmune sera specific for RNAP II (data not shown). Finally, in pulse-chase experiments, these sera immunoprecipitated the 145-kD subunit after pulse labeling, followed by immunoprecipitation of both the 145 and the phosphorylated (Ilo) form of the large subunit 2–4 h later. It is unlikely that this pattern simply reflects recognition of the 145-kD subunit, because the Ilo and Ila forms of RNAP II both carry the 145-kD subunit (33), and the sera do not immunoprecipitate the Ila form. Interpretation of the pulse-chase experiments must take into account the fact that the immunoprecipitation patterns reflect the kinetics of phosphorylation and subunit assembly, as well as the epitope(s) recognized by autoantibodies. Phosphorylation of the Ila subunit and particle assembly occur at ~2–4 h after de novo synthesis of RNAP II subunits (Fig. 3). Taking these kinetic factors into account, there are at least three models that might explain why some sera specific for RNAP Ilo immunoprecipitated only the 145-kD (Ilo) subunit after pulse labeling (Fig. 4). One possible explanation is that the sera may recognize an epitope of the 145-kD subunit that is covered when the Ila subunit binds, but is exposed by time-dependent, phosphorylation-induced, conformational changes (38) (Fig. 4 A). Alternatively, some sera may contain antibodies specific for the phosphorylated (Ilo) form of the large subunit, and the newly synthesized (labeled) 145-kD subunit may form complexes rapidly with preexisting (unlabeled) Ilo subunits (Fig. 4 B). In this scenario, the newly synthesized Ila subunit would not be recognized until it is phosphorylated 2–4 h later, thus explaining the immunoprecipitation of the labeled 145-kD subunit, but not the large subunit, after pulse labeling. Finally, it is possible that these sera contain antibodies to a conformation unique to the complex of the Ilo and the 145-kD subunits (Fig. 4 C). Direct binding of these two subunits has been reported (27), and it has been suggested that phosphorylation of the Ila subunit causes a major change in the conformation of RNAP II (38). This conformation might be recognized by autoantibodies immunoprecipitating only the Ilo form of RNAP II. Autoantibodies specific for conformational epitopes formed by multiprotein or protein–nucleic acid complexes have been described previously, e.g., in the case of anti-

nucleosome antibodies and autoantibodies specific for the human Y5 Ro ribonucleoprotein particle (39, 40). Further experiments will be necessary to distinguish between these possibilities. Unfortunately, Western blot analysis did not resolve the question of subunit specificity due to the poor reactivity of these sera with RNAP II subunits after SDS-PAGE and transfer to nitrocellulose (M. Satoh, unpublished data). This may further support the idea that some autoantibodies recognize a conformational epitope unique to the IIO form of RNAP II. Regardless of the explanation, these sera appear to recognize RNAP II in a manner distinct from that displayed by other autoimmune sera and mAb 8WG16, which immunoprecipitate only the unphosphorylated form of the large subunit after pulse labeling, followed by coimmunoprecipitation of the 145-kD subunit.

The specific recognition of the phosphorylated (IIO) form of RNAP II by over half of the sera suggests that autoimmunity may be directed selectively against the RNAP II elongation complex, since phosphorylation of RNAP II is thought to occur in the preinitiation complex and is required for elongation to take place (32, 41, 42). Autoantibodies specific for the phosphorylated form of the large subunit may be of some interest in view of previous suggestions that autoantibodies target active or functional sites preferentially (43, 44), and the evidence that phosphorylation of the large subunit is characteristic of the transcriptionally active form of RNAP II (38, 41, 42, 45). It has been shown previously that human autoantibodies that immunoprecipitate RNAP II can inhibit the catalytic activity of the enzyme (12). However, it remains to be determined whether autoantibodies specific for the phosphorylated form of the enzyme can likewise inhibit function.

Diagnostic specificity of anti-RNAP antibodies. In the present study, anti-RNAP II antibodies were detected in 9% of Japanese, 7% of Caucasian, and 18% of Black SLE patients who did not show manifestations of other rheumatic diseases. Autoantibodies to RNAP II in SLE and MCTD/overlap syndromes were rarely associated with anti-RNAP I/III antibodies, consistent with previous observations that the latter are specific for SSc (11–15). The single SLE patient with anti-RNAP I/III antibodies is the first such patient reported, suggesting that autoantibodies to RNAP I/III are extremely unusual in SLE. In addition, in contrast to previous reports, autoantibodies to RNAP II without anti-RNAP I/III antibodies were also detected frequently in SSc.

Kuwana et al. (14) reported previously that antibodies to RNA polymerases were not found in 286 controls including 190 SLE and 10 SLE-PM overlap syndrome, although anti-RNAP I, II, III antibodies were analyzed together, and detailed analysis of the specificities was not performed. Hirakata et al. (12) specifically looked at anti-RNAP II and reported that anti-RNAP II antibodies were not detected in sera from 217 controls, including 126 SLE and 27 MCTD patients. The discrepancy between our data and these previous reports is probably not explained entirely by differences in the patient populations tested, because autoantibodies to RNAP II were found in Japanese as well as American patients in the present study. It is possible that autoantibodies to RNAP II were detected more frequently when accompanied by anti-RNAP I/III, and that antibodies to RNAP II alone were underreported due to a lower labeling efficiency or differences in the extraction buffers used for immunoprecipitation. Also, anti-RNAP II autoantibodies specific for the phosphorylated form of the largest subunit, which were present in more than half of patients with anti-

RNAP II in the present study, might have been overlooked previously.

Association of anti-RNAP II and other autoantibodies. In contrast to previous suggestions that anti-RNAP antibodies seldom coexist with other marker autoantibodies for SSc subsets (14, 15, 35), we found that anti-RNAP II antibodies were often accompanied by anti-nRNP, anti-Ku, or anti-topoisomerase I antibodies. However, sera containing all three of these specificities along with anti-RNAP II were not identified. Further studies with larger numbers of patients will be necessary to verify the significance of the associations of anti-RNAP II with other autoantibody specificities. If confirmed by more extensive analysis, the coexistence of anti-RNAP II antibodies with anti-Ku and anti-topoisomerase I antibodies may be of interest from both biological and immunological points of view. RNAP II is an *in vitro* substrate for phosphorylation by the Ku-p350 DNA-dependent protein kinase complex (46). Likewise, there is recent evidence that topoisomerase I is a cofactor for transcription by RNAP II and that it interacts physically with both RNAP II and the TATA binding protein (47, 48). An association of autoantibodies to RNAP II with anti-Ku or anti-topoisomerase I antibodies might, therefore, be consistent with the concept of linked sets of autoantibodies specific for physically associated components of a macromolecular complex (49). Alternatively, anti-RNAP II, anti-Ku, anti-topoisomerase I, and probably anti-nRNP antibodies may all be associated with the same clinical subset, or reactivity with multiple antigens might reflect autoantibody recognition of shared epitopes. Studies with larger numbers of patients are necessary to confirm the true associations of anti-RNAP II autoantibodies with other specificities and to explain these associations.

In summary, we report here that anti-RNAP II antibodies are common in SLE and MCTD/overlap syndrome and are not a disease marker for SSc, whereas anti-RNAP I/III antibodies appear highly specific for SSc. In over half of the patients, the autoantibodies were specific for the transcriptionally active phosphorylated form of the enzyme. Whether particular anti-RNAP II antibodies have other clinical significance, such as an association with specific symptoms or prognosis, may be clarified in future studies. Further investigation of the immunologic basis for the possible linkage of anti-RNAP II antibodies with other autoantibodies may help in understanding why certain autoantibodies serve as specific markers for clinical subsets of rheumatic disease.

Acknowledgments

We are grateful to Dr. Nancy E. Thompson (McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI) for providing an mAb specific for RNAP II, and to Dr. Yutaka Okano and Dr. Thomas Medsger (University of Pittsburgh, PA) for supplying human reference sera. We would like to thank Drs. Mary Anne Dooley, Philip L. Cohen, Donald Kimpel, and Michael Maldonado (University of North Carolina, Chapel Hill, NC), and Jules Hirsch (Rockefeller University, New York, New York) for assistance in obtaining clinical information. The assistance of Dr. John B. Winfield, Mr. Brad Marcum, and Ms. Robin Roseberry in establishing and maintaining the computerized clinical and serological databases used in these studies is also gratefully acknowledged.

This work was supported by grants RO1-AR40391, P50-AR42573, P60-AR30701, T32-AR7416, and RR00046 from the United States Public Health Service. Dr. Wang is the recipient of a postdoctoral fellowship from the Arthritis Foundation.

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