# Antibodies to RNA Polymerase III in Systemic Sclerosis Detected by ELISA

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***ABSTRACT.*** ***Objective.*** To determine serological and clinical variables associated with anti-RNA polymerase III (RNAP-III) antibodies in patients with systemic sclerosis (SSc) using a new ELISA method.

***Methods.*** Sera from 242 patients with SSc were collected from 14 Canadian clinics. Control sera were from 287 blood donors, and 42 patients with infectious disease, 30 with rheumatoid arthritis (RA), and 30 with systemic lupus erythematosus (SLE). Antibodies to RNAP-III were detected by an ELISA kit and antibodies to other cellular antigens were identified by indirect immunofluorescence (IIF) on HEp-2 cell substrate, line immunoassay, immunoprecipitation of recombinant protein, and addressable laser bead immunoassay (ALBIA).

***Results.*** Anti-RNAP-III antibodies were detected in 47/242 (19.4%) SSc sera, 0% RA and SLE sera, 1/287 blood donor sera, and 2/42 infectious disease sera. Diffuse disease (59.5%) was more common than limited disease (36.1%) in the anti-RNAP-III-positive patients ($p = 0.006$) and there was an association between the presence of anti-RNAP-III and kidney and joint/tendon involvement, but there was no association with a nucleolar IIF pattern, lung involvement, or other clinical indicators. There was a negative association between the presence of anti-RNAP-III antibodies and anticentromere by IIF ($p = 0.00004$) and anti-Scl-70 by ALBIA ($p = 0.0005$) and line immunoassay ($p = 0.003$), suggesting a virtually exclusive presence of these antibodies in SSc.

***Conclusion.*** Anti-RNAP-III autoantibodies were found in nearly 20% of SSc patients but in less than 1% of controls, thus detection of this antibody is a useful marker to help diagnose SSc. As well, this antibody has prognostic utility, since it is associated with scleroderma renal crisis and the diffuse cutaneous form of SSc. (First Release June 15 2007; J Rheumatol 2007;34:1528–34)

*Key Indexing Terms:*
AUTOANTIBODIES SYSTEMIC SCLEROSIS RNA POLYMERASE ELISA

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Autoantibodies to RNA polymerase (RNAP) were first reported in 1982[1], and in 1987 the specificity of this reaction was refined to show that these antibodies targeted RNAP-I[2]. Since then, it has been shown that antibodies also target RNAP-III and are found in 4%–25% of SSc sera, and are associated with the diffuse cutaneous form of SSc (dcSSc) and an increased risk of kidney and cardiac involvement[3-8]. Although antibodies to RNAP-II and RNAP-I have also been described in a variety of diseases, RNAP-I antibodies are rarely observed in conditions other than SSc[9].

Until recently, the detection of anti-RNAP antibodies was performed by time-consuming immunoprecipitation (IP) assays. Recently, ELISA kits that employ a recombinant RNAP-III fragment[10] have become available and have been employed in studies to evaluate the clinical and serological measures associated with these autoantibodies[11,12]. Because the assay employing this purified antigen is highly specific for SSc, it is likely that it will find a useful clinical application. Since RNAP-I antibodies nearly always coexist with RNAP-III antibodies[3,4,12], it is thought that an anti-RNAP-III assay would serve as a useful surrogate for both autoantibodies. Although early studies suggested that RNAP autoantibodies might be correlated with a speckled nucleo-

lar pattern of indirect immunofluorescent (IIF) staining, a recent study found that antibodies to RNAP-III were not consistently associated with this IIF pattern on conventional HEp-2 cell substrates[12]. Indeed, sera with anti-RNAP-III more consistently had a nuclear speckled IIF pattern[3,7], a finding consistent with observations that monoclonal anti-RNAP-III antibodies stain nuclei, whereas antibodies to RNAP-I stain nucleoli[2]. Therefore, while the clinical usefulness of detecting anti-RNAP-III has been demonstrated, it cannot be assumed that IIF on conventional HEp-2 cell substrates serves as a useful screen to detect these antibodies.

We investigated the prevalence and serological and clinical associations of anti-RNAP-III measured by a new commercial ELISA in an unselected SSc cohort and various control populations, and also determined the sensitivity and specificity of the assay for the diagnosis of SSc.

## MATERIALS AND METHODS

*Sera and patient enrollment*. Sera were obtained from most SSc patients seen by the Canadian Scleroderma Research Group. The group consists of 15 rheumatologists in Canada who see patients with SSc once a year and enter clinical and laboratory data onto standardized forms. Patients also complete questionnaires and standardized instruments such as the Health Assessment Questionnaire (HAQ), the Medical Outcomes Study Short Form-36 (SF-36), and an assessment of severity using the severity scale of Medsger, *et al*[13] as described below. The study had the approval of the ethics review board of each participating center and each patient signed a consent form on enrollment.

*Disease subsets*. Patients were classified as having limited cutaneous SSc (lcSSc) on the basis of skin thickening distal to the elbows and knees throughout their time of clinical observation. Skin thickening limited to the facial area was also permitted in lcSSc. Patients were classified as having dcSSc when they had skin thickening proximal to the elbows or knees (affecting upper arms, thighs, or trunk) at any time during clinical observation.

*Disease characterization*. The Scleroderma Disease Severity Score (DSS) was adapted from Medsger, *et al*[13]. A severity score of 0 (normal) to 4 (end-stage) was generated for each of the 9 systems. The worst category was scored for each system and results of any investigation not requested by the physician, therefore missing, were considered "normal." Scoring methods for General, Peripheral Vascular, Skin, and Joint/tendon, Lung, and Kidney organ systems were identical to those proposed by Medsger, *et al*[13]. Some adaptations were made to the other organ system evaluations (Table 1). In order to assign a score for the Skeletal Muscle system, physicians were asked to rate patients' muscle strength in 5 different areas of the body (neck flexors and upper and lower proximal extremities, right and left) using the British Medical Research Council scale[14]. A severity score was then assigned depending on the total number of scores of 5, 4, 3, 2, 1, and 0 for a given patient (see details under Muscle, Table 1). The HAQ-Damage Index (HAQ-DI) was used to assess the patient's use of ambulation aids needed to assign the worse severity level for the Skeletal Muscle system (i.e., level 4, Endstage). To score the gastrointestinal system in addition to an abnormal esophagram, abnormal esophageal manometry, or abnormal small bowel series, patients reporting difficulty swallowing, acid taste in the mouth, choking at night, burning sensation, feeling of being full shortly after eating, or taking gastroprotective or promotility agents were also given a score of 1 (Mild). In addition to malabsorption syndrome and episodes of pseudo-obstruction, patients with an abnormal hydrogen breath test were given a score of 3 (Severe). To score the Heart system, electrocardiogram results, left ventricular ejection fraction (%) values, the presence of conduction abnormalities, distended neck veins, and arrhythmia diagnosed by a physician were used. For the purposes of this study, a score ≥ 1 for a system indicated involvement.

*Laboratory procedures*. Aliquots of sera were stored at –70°C until needed and all autoantibody testing was performed at the Advanced Diagnostics Laboratory, University of Calgary. This included an autoantibody analysis by IIF on HEp-2000 substrate (ImmunoConcepts Inc., Sacramento, CA, USA), autoantibodies to scleroderma-related autoantigens [CENP-B, Scl-70 (topo-I), PM/Scl, and fibrillarin–Scl-34; Mikrogen, Neuried, Germany], antibodies to PM/Scl detected by ELISA using a commercial PM1-α antigen (amino acids 231–245 of the PM/Scl-100; Dr. Fooke Laboratorien GmBh, Neuss, Germany), antibodies to fibrillarin by a TnT immunoprecipitation technique, and antibodies to extractable cell antigens by addressable laser bead immunoassay (ALBIA; QuantaPlex SLE Profile 8, INOVA Diagnostics, San Diego, CA, USA).

Control sera were from 288 blood donors, 42 samples with high titers of antibodies to markers of infectious diseases, and 30 patients diagnosed with rheumatoid arthritis (RA), all purchased from SeraCare Diagnostics (Milford, MA, USA) or ProMedix (Union City, NJ, USA). Sera of 30 patients with systemic lupus erythematosus (SLE) were from an established serum library.

*Anti-RNAP-III antibodies by ELISA*. An ELISA kit (INOVA Diagnostics) was used to detect anti-RNAP-III as suggested by the manufacturer. The antigen is the immunodominant epitope of RNAP-III described by Kuwana, *et al*[10,11] that has been cloned in a baculovirus vector and expressed in sf9 cells. Patient samples were diluted 1/100 and then added to each well. Horseradish peroxidase coupled to anti-human IgG conjugate supplied with the kit was used as the secondary antibody. After developing the reaction with the chromogen supplied, the absorbance was read at 450 nm. Values < 20 U were considered negative, and positive results were classified as weak (20–39 U), moderate (40–80 U), and strong (> 80 U), as suggested by the manufacturer.

*Statistical analysis*. All clinical data were entered into and extracted from SPSS for Windows (version 14.0) maintained at McGill University, Montréal. Some results were expressed as mean ± standard deviation. T test was used to compare means. The association between qualitative variables was evaluated by chi-square corrected (Yates) or Fisher exact test, when indicated, considering $p < 0.05$ as statistically significant.

## RESULTS

*Anti-RNAP-III antibodies by ELISA*. When the ELISA method was used to detect anti-RNAP-III antibodies in the SSc sera, a prevalence of 19.4% (47/242) was observed (Figure 1). By comparison, anti-RNAP-III antibodies were not found in the 30 RA or 30 SLE sera, but were found in 1/288 (0.3%) blood donor samples and 2/42 (5%) of infectious disease controls (Table 2). Both of the positive patients with infectious disease had low levels of reactivity (22 units) and both had cytomegalovirus infection.

*Demographic data of SSc patients*. The unselected sera included in this study were from 242 patients with SSc enrolled by participating centers across Canada. These included 214 (88.5%) women and 28 (11.5%) men, with a mean age of 56 (± 12) years. The majority of the patient population was Caucasian (83.1%). One hundred thirty-nine patients had lcSSc (57.4%), 95 had dcSSc (39.3%), and 8 (3.3%) were not classified as either SSc subset. The mean duration of the disease from the first non-Raynaud manifestation was 10.3 (± 8.4) years (Table 3). In addition, there was no statistically significant difference in the frequency of

*Table 1*. Adaptations to the Systemic Sclerosis Disease Severity Scale of Medsger, *et al*[13].

| Organ System | 0<br>Normal | 1<br>Mild | 2<br>Moderate | 3<br>Severe | 4<br>Endstage |
|---|---|---|---|---|---|
| Skeletal/muscle: 5 areas of body scored according to British Medical Research Council[14] | ≤ 1 area grade 4, others = grade 5 | 2 areas grade 4 AND others = grade 5 | ≥ 3 areas grade 4 OR 1 area grade 3 AND 1 area grade 2 AND ≥ 3 areas grade 4 | ≥ 2 areas 3 OR ≥ 2 grade 2 OR one or more grade 1 | Level 2 (moderate) OR level 3 (severe) AND ambulation aids on HAQ |
| Gastrointestinal tract | Barium swallow normal or not done OR esophageal monometry normal or not done OR small bowel normal or not done | Barium swallow abnormal OR presence of distal esophageal OR small bowel abnormal OR according to patient: difficulty swallowing OR acid tasting in mouth OR choking at night OR burning sensation OR being full OR taking gastroprotective or promotility agents | Antibiotics for bacterial overgrowth | Malabsorption syndrome OR episodes of pseudo-obstruction OR abnormal hydrogen breath test | Hyperalimentation |
| Heart | Normal EKG OR no arrhythmia according to physician OR LVEF ≥ 50% | Conduction abnormalities OR mild arrhythmia according to physician OR LVEF 45–49% | Arrhythmia on EKG OR moderate arrhythmia according to physician OR LVEF 40–44% | Severe arrhythmia according to physician OR LVEF 30–39% | Distended neck vein OR LVEF < 30% |

LVEF: left ventricular ejection fraction.

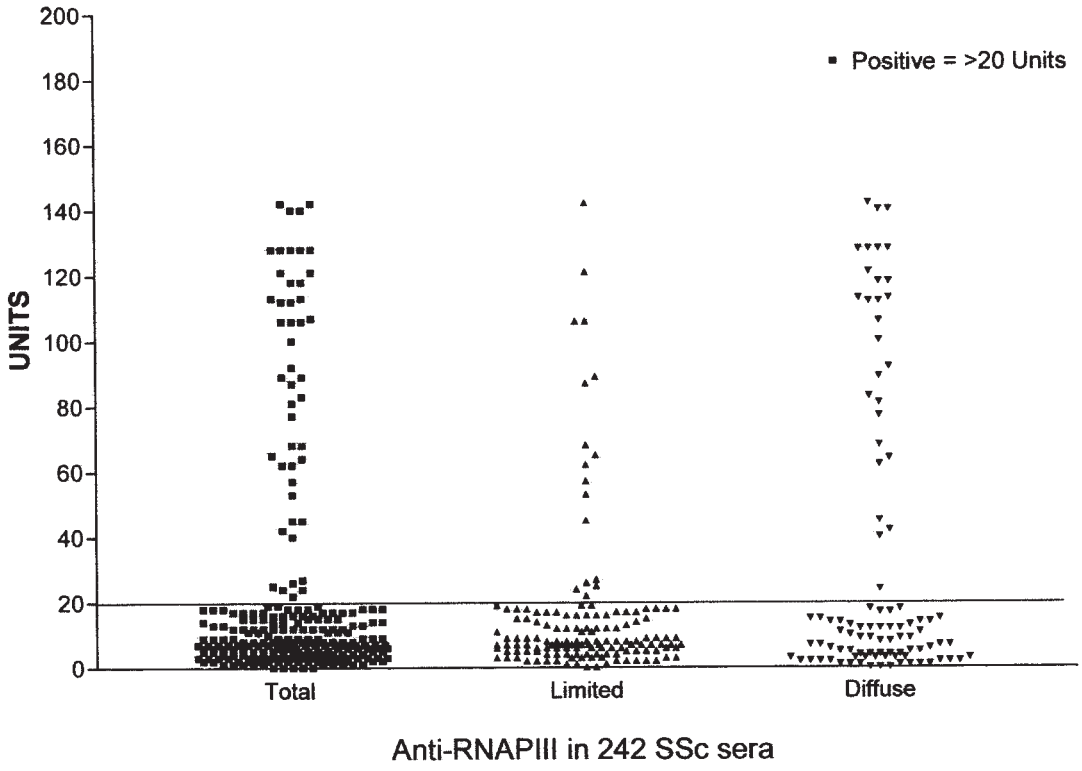


*Figure 1*. Frequency of anti-RNAP-III antibodies in 242 Canadian SSc patients as detected by ELISA (data are expressed in units as recommended by the manufacturer). dcSSc was more common than lcSSc in the anti-RNAP-III-positive patients (p = 0.006).

*Table 2*. Frequency of anti-RNAP-III in patients with SSc and controls.

| Disease | Anti-RNAP-III, n (%) |
|---|---|
| Canadian SSc | 47/242 (19.4) |
| Rheumatoid arthritis | 0/30 (0) |
| Systemic lupus erythematosus | 0/30 (0) |
| Infectious diseases | 2/42 (5) |
| Herpes simplex | 0/8 (0) |
| Hepatitis C | 0/8 (0) |
| Cytomegalovirus | 2/9 (22) |
| Parvovirus | 0/7 (0) |
| Rubella | 0/4 (0) |
| Toxoplasmosis | 0/6 (0) |
| Blood donors | 1/288 (0.3) |
| All non-SSc combined | 3/390 (0.8) |

anti-RNAP-positive or negative results in the 45 French Canadian patients in this cohort compared to the rest of the cohort (Pearson's chi-square = 0.468; p = 0.4936). Other clinical features based on the adapted Medsger severity scale are shown in Table 4.

*Clinical and serological correlates of anti-RNAP-III antibodies*. The comparative clinical data for anti-RNAP-positive and anti-RNAP-negative patients are presented in Tables 3 and 4. Among anti-RNAP-III-positive patients, 59.5% had diffuse and 36.1% had limited disease (Table 3). By contrast, among anti-RNAP-III-negative patients, 62.5% had lcSSc and 34.3% had dcSSc. Thus, diffuse disease was more common in the anti-RNAP-III-positive patients (p = 0.006 by chi-square) and lcSSc was more frequent in the anti-RNAP-III-negative group (p = 0.002 by chi-square). There was an association between the presence of anti-RNAP-III and "kidney disease" and "joint and tendon" system as evaluated by the Medsger severity scale, but there was no association between these antibodies and lung involvement or other clinical variables (Table 4). Such an association was not observed even when we analyzed the subgroup with more severe lung disease, that is, lung severity score ≥ 2. Although a nucleolar IIF pattern of staining was seen in 98/240 (40.8%) of the SSc sera, only 17/98 (17.3%) had anti-RNAP-III and only 17/47 (36%) anti-RNAP-III sera had a nucleolar IIF pattern, a relationship that was not statistically significant (p = 0.57). The frequency of other autoantibodies in the 242 samples (Table 5) included 68/237 (28.7%) with anticentromere protein (CENP) determined by IIF as compared to the line immunoassay (LIA) result of 82/234 (35%). Anti-PM/Scl was found in 17/234 (7.3%) by ELISA and 13/234 (5.6%) by LIA. In order of decreasing frequency, other autoantibodies included anti-topo-I (22.5% by ALBIA and 22.6% by LIA), anti-SSA/Ro (22.1%), antichromatin (11%), anti-U1-RNP (9.2%), anti-Sm (4.6%), anti-SSB/La (3.8%), and anti-fibrillarin (2.8%).

*Table 3*. Demographic features and classification of Canadian patients with SSc.

| | Entire Cohort, n = 242 | RNAP III-Positive, n = 47 | RNAP III-Negative, n = 195 | p |
|---|---|---|---|---|
| Age, mean ± SD yrs | 56.3 (± 12.5) | 57.5 (± 11.9) | 56 (± 12.6) | 0.998 |
| Caucasoid, % | 83.1 | 82.9 | 83 | 0.987 |
| Female, % | 88.5 | 84.4 | 89.5 | 0.335 |
| Disease duration, mean ± SD yrs | 10.3 (± 8.4) | 8.1 (± 6.2) | 10.9 (± 8.8) | 0.048 |
| lcSSc (%) | 139 (57.4) | 36.1 | 62.5 | 0.001 |
| dcSSc (%) | 95 (39.3) | 59.5 | 34.3 | 0.001 |
| Unclassified (%) | 8 (3.3) | 4.2 | 3.0 | 0.655 |

*Table 4*. Organ system involvement. Organ involvement was said to be present if the patient was ascribed a score ≥ 1 on the adapted version (Table 1) of the severity scale of Medsger, *et al*[13].

| Organ System | Entire SSc Cohort, (n = 242), % | RNAP III-Positive, (n = 47), % | RNAP III-Negative, (n = 195), % | p |
|---|---|---|---|---|
| General | 51.6 | 51.1 | 51.6 | 1.0 |
| Skin | 94.3 | 97.8 | 93.3 | 0.316 |
| Kidney | 7.1 | 24.3 | 3.1 | < 0.001 |
| Peripheral vascular | 70 | 68.8 | 70.3 | 0.857 |
| Joint/tendon | 52.8 | 74.4 | 47.6 | 0.001 |
| Muscle | 12.2 | 18.6 | 10.7 | 0.194 |
| Gastrointestinal | 91.3 | 89.3 | 92.3 | 0.555 |
| Lung | 74.3 | 78.7 | 73.3 | 0.577 |
| Heart | 32.2 | 29.7 | 32.8 | 0.732 |

*Table 5*. Association between anti-RNAP-III and other autoantibodies in 242 SSc sera.

| Autoantibodies | Immunoassay | Anti-RNAP-III-Positive, n = 47 (%) | Anti-RNAP-III-Negative, n = 195 (%) | p |
|---|---|---|---|---|
| CENP | IIF | 3/47 (6.3) | 66/190 (34.2) | 0.00004 |
| CENP B | LIA | 6/43 (13.9) | 76/191 (39.7) | 0.002 |
| Chromatin | ALBIA | 3/45 (6.6) | 23/192 (11.9) | 0.22 |
| PM/Scl | ELISA | 1/42 (2.3) | 16/192 (8.3) | 0.15 |
| PM/Scl | LIA | 1/43 (2.3) | 12/191 (6.2) | 0.27 |
| Fibrillarin | IP | 0 | 7 (2.8) | |
| U1-RNP | ALBIA | 2/47 (4.2) | 20/192 (10.4) | 0.15 |
| Topo-I | ALBIA | 2/44 (4.5) | 51/192 (26.5) | 0.0005 |
| Topo-I | LIA | 3/43 (6.9) | 50/191 (26.1) | 0.003 |
| Sm | ALBIA | 2/46 (4.3) | 9/191 (4.7) | 0.63 |
| SSA/Ro | ALBIA | 9/44 (20.4) | 43/191 (22) | 0.92 |
| SSB/La | ALBIA | 1/47 (2.1) | 8/193 (4) | 0.44 |

ALBIA: addressable laser bead immunoassay; CENP: centromere protein; ELISA: enzyme linked immunoassay; IIF: indirect immunofluorescence; IP: immunoprecipitation; LIA: line immunoassay; PM: polymyositis; RNP: ribonucleoprotein; Scl: scleroderma; Sm: Smith antigen; Topo-I: topoisomerase I.

The presence of anti-RNAP-III antibodies was not statistically associated with antibodies to other autoantigens such as Sm, RNP, SSA/Ro, SSB/La, or chromatin. On the other hand, there was a statistically significant negative association between the presence of anti-RNAP-III antibodies and anti-CENP-B by LIA (p = 0.002), anticentromere by IIF (p = 0.00004), and anti-topo-I by ALBIA (p = 0.0005) and LIA (p = 0.004). Antibodies to PM/Scl were found in only one and anti-fibrillarin antibodies were found in none of the patients with anti-RNAP-III; however, the number of positive samples for these antibodies was very small.

## DISCUSSION

A new commercially available ELISA detected anti-RNAP-III in 19.4% of an unselected cohort of Canadian patients with SSc. The presence of these antibodies was primarily associated with the dcSSc and the development of kidney and joint/tendon involvement. In one of the earliest studies, antibodies to RNAP-III detected by immunoprecipitation were found in 57/252 SSc patients (23%; 95% CI 18%–28%) including 50/111 (45%) who had dcSSc and 7/114 (6%) who had lcSSc[4]. In this same study, patients with anti-RNAP-III antibody had a statistically significantly higher mean maximum skin thickness score but statistically significantly lower frequencies of telangiectasias, inflammatory myopathy, restrictive lung disease, and serious cardiac abnormalities than patients with anti-topo-I[4]. In another study of a cohort of Caucasian patients with connective tissue disease that used both ELISA and IP of $^{35}$S-labeled HeLa cell extracts, anti-RNAP antibodies were detected in 8/36 (22%) Caucasian patients with dcSSc and 1/53 (2%) with lcSSc[16]. Another study of anti-RNAP antibodies suggested that they were associated with diffuse cutaneous involvement and male sex[17]. A more recent study used an anti-RNAP-III ELISA in patients with SSc and found an association with dcSSc, a higher maximum total skin score, and increased frequency of tendon friction rubs[11].

A study of Japanese, American Caucasian, and American Black patients with SSc found that the highest frequency of anti-RNAP was in the American Caucasian group[18]. A recent international study by Meyer, *et al* found that the frequency of anti-RNAP-III measured by IP in French patients with SSc was only 4%, in contrast to 25% in American patients[8]. It was suggested that this difference might be due to genetic factors that discriminate French from American patients. Although a proportion of our patients are of French Canadian heritage, we found no difference in the frequency of anti-RNAP in this subset. Thus, the frequency of anti-RNAP in our study is more comparable to the reported American frequency. In addition, it was suggested that there might be a north-south gradient that would explain the different frequencies of RNAP-III antibodies in Europe. That the frequency of anti-RNAP in Canadian SSc is not remarkably different from the American SSc reported by Meyer, *et al*[8] suggests that such a gradient does not exist in North America. Thus, the frequency of anti-RNAP-III antibodies identified in our study and the clinical correlation with dcSSc and renal and joint/tendon involvement is in agreement with the few other studies that have looked at these variables. We did not evaluate and compare the ELISA with respect to other immunoassays, such as IP, although studies in other centers found excellent concordance between these 2 assays[10,11,16].

Earlier studies suggested that sera containing antibodies to RNAP-I and III were exclusive of anti-CENP and anti-topo-I, and formed a comparatively distinct serological subgroup (7.8%–11.7%)[7,17]. In our study, antibodies to RNAP-III were found in a few sera that contained antibodies to CENP (6 by LIA) and topo-I (3 by LIA), but not in sera that contained anti-fibrillarin, and only one had anti-PM/Scl antibodies. Thus, although a few sera with both anti-RNAP-

III and other SSc "marker" antibodies were observed, our data support the previous conclusions that these autoantibodies tend to be mutually exclusive. However, some sera with anti-RNAP-III antibodies did have other concurrent antibodies. In this regard, antibodies to SSA/Ro were the most common, at 20.4%, a frequency similar to that reported in another RNAP-III cohort, 21%[19].

It is interesting that there was a statistically significant negative association between the presence of anti-RNAP-III antibodies and other scleroderma-specific autoantibodies such as anti-CENP-B and anti-topo-I. Although we found a higher frequency of overlap in these antibodies than previous studies (5% with anti-topo-I and 12% with CENP), this is likely due to more sensitive assays (ALBIA and LIA) used in our study or differences in the makeup of the SSc cohort we studied.

It is also notable that only 2 (4.2%) RNAP-III-positive serum samples had antibodies to U1-RNP as compared to a frequency of 10.4% in the RNAP-III-negative group. Our findings are also in agreement with other reports that antibodies to chromatin are found in < 15% of SSc sera[20-23]. Of interest, there was generally good agreement between the various assays we used in the detection of topo-I and PM/Scl antibodies.

Many of the sera with anti-RNAP-III antibodies displayed an IIF pattern of nuclear fine speckles with a few discrete dots, as described previously[7]. However, in agreement with a recent study[12], some of the sera with anti-RNAP-III antibodies displayed a nucleolar IIF pattern, even though the majority of sera with a nucleolar IIF pattern were negative for anti-RNAP-III antibodies. Thus, the IIF pattern is not a sensitive marker for anti-RNAP-III. In the appropriate clinical setting, anti-RNAP-III antibodies should be sought, irrespective of the IIF pattern. Other studies have suggested that a punctate nucleolar staining pattern may provide a clue to the presence of anti-RNAP-I, whereas a clumpy nucleolar staining pattern is more indicative of antibodies to fibrillarin or Th[2,24]. It is possible that the 14 sera with a nucleolar pattern in our study had concomitant anti-RNAP-I antibodies, but we did not perform assays for anti-RNAP-I. It has been shown that anti-RNAP-III always coexist with anti-RNAP-I[3,4]. In addition, our studies of the Canadian scleroderma cohort have been unable to confirm that the pattern of nucleolar staining is highly correlated with fibrillarin or PM/Scl antibodies (unpublished data).

A new ELISA was shown to detect anti-RNAP-III antibodies in roughly 20% of an unselected Canadian SSc cohort. Since most sera with anti-RNAP-III antibodies segregate independently while being specific for the diagnosis of SSc, the anti-RNAP-III assay is a valuable addition to serological tests used in the diagnosis of SSc. The presence of these antibodies may also have prognostic utility, since they were correlated with the dcSSc and with increased prevalence of renal and joint/tendon disease.

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