

Nucleolar Staining Cannot Be Used as a Screening Test for the Scleroderma Marker Anti-RNA Polymerase I/III Antibodies

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Objective. Anti-RNA polymerase I/III (anti-RNAP I/III) antibodies are clinically useful markers of scleroderma, and their presence is associated with diffuse skin disease and an increased risk of cardiac and kidney involvement. Although RNAP I antibodies localize to the nucleolus, nucleolar staining by many anti-RNAP antibody-positive sera is not always observed. Nucleolar staining by anti-RNAP antibody-positive sera was examined by double staining with antifibrillarin antibodies to evaluate whether nucleolar staining can be used as a screening test for anti-RNAP I/III antibodies. In addition, the relationships between nucleolar staining and levels of anti-RNAP III antibodies were examined by enzyme-linked immunosorbent assay (ELISA) and immunoprecipitation (IP) assay.

Methods. Sera were tested using immunofluorescent antinuclear antibodies on HEp-2 cell slides, by anti-RNAP III ELISA, and by IP assay using ³⁵S-labeled K562 cell extract. Nucleolar staining by anti-RNAP antibody IP-positive sera was confirmed by double staining using antifibrillarin monoclonal antibodies. The levels of anti-RNAP III antibodies were quantitated by ELISA and by IP assay using a serially diluted reference serum as a standard, and their relationship was analyzed.

Results. All 18 anti-RNAP I/III antibody-positive sera showed nuclear speckled patterns, but nucleolar

staining was readily noticeable in only 44% of the sera. A positive correlation was found between ELISA and IP units for anti-RNAP III antibodies. The levels of anti-RNAP III antibodies and anti-RNAP I antibodies correlated well, with the exception of a few sera. Levels of anti-RNAP III antibodies were low in sera with nucleolar staining, whereas several sera with high levels of anti-RNAP I antibodies clearly showed nucleolar staining.

Conclusion. Although some sera positive for anti-RNAP I/III antibodies clearly stain nucleoli, nucleolar staining is inconsistent and cannot be used to screen for anti-RNAP I/III antibodies.

Autoantibodies associated with scleroderma (systemic sclerosis [SSc]), such as anti-topoisomerase I antibodies, anti-RNA polymerase I/III (anti-RNAP I/III) antibodies, and anticentromere antibodies, are not only helpful in establishing the diagnosis, but also serve as a clinically useful marker for predicting specific organ involvement and outcome (1). However, with the exception of anticentromere antibodies, which can be identified by immunofluorescent antinuclear antibodies (ANAs), and anti-topoisomerase I antibodies, which can be identified by commercially available enzyme-linked immunosorbent assays (ELISAs), tests for SSc antibodies are not available.

Anti-RNAP I/III antibodies, which are found in ~20% of patients with SSc, have been established as a disease marker associated with diffuse scleroderma and an increased risk of cardiac and kidney involvement (1–4). An anti-RNAP III ELISA using a recombinant fragment as an antigen (5) became commercially available in 2004. Because this is a clinically useful test with excellent sensitivity and specificity (5,6), it will be used more frequently in the future. Anti-RNAP III antibodies always coexist with anti-RNAP I antibodies (2,3),

Supported by NIH grants R01-AR-40391 and M01-R-00082.

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Submitted for publication March 7, 2006; accepted in revised form May 16, 2006.

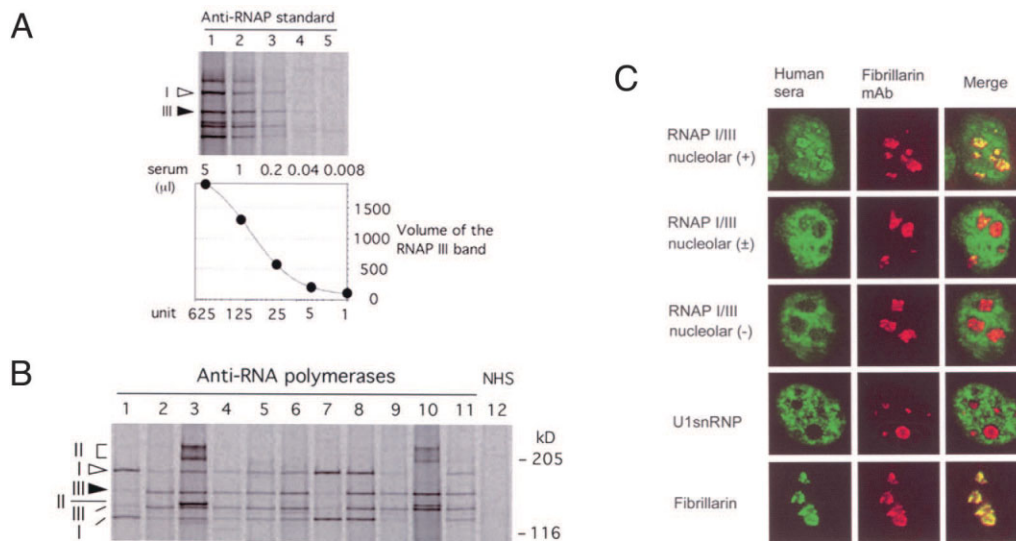


Figure 1. **A**, Immunoprecipitation assay using serially diluted standard serum, with conversion into units. Extracts from ^{35}S -labeled K562 cells were immunoprecipitated using a serially diluted standard serum with high levels of anti-RNA polymerase (anti-RNAP) antibodies. A phosphorimage of the autoradiogram is shown (top). A standard curve was made based on the volume of the largest subunit of RNAP III (black arrowhead). An RNAP I standard curve was made in the same way, using the volume of the largest subunit of RNAP I (white arrowhead). **B**, Immunoprecipitation of anti-RNAP antibody-positive sera for quantification. ^{35}S -labeled K562 cell extract was immunoprecipitated using 1 μl of serum. The levels of anti-RNAP III antibody (black arrowhead) or anti-RNAP I antibody (white arrowhead) were estimated based on the volume of the largest subunit bands from the phosphorimage, converted into units using the standard curve (**A**). **C**, Immunofluorescent antinuclear antibodies. HEP-2 cells were stained with human systemic sclerosis sera positive for anti-RNAP I/III antibodies, anti-U1 small nuclear RNP (anti-U1 snRNP), or antifibrillarin (left column, green), with double staining using mouse monoclonal antibodies to fibrillarin (middle column, red). Merged images show yellow color for colocalization of green and red fluorescence (right column). Nucleolar staining was apparent in some anti-RNAP I/III antibody-positive sera (+). Less clear nucleolar staining in some sera was confirmed by the merged image (\pm). Some anti-RNAP I/III antibody-positive sera were completely negative with double staining (-). Sera positive for anti-U1 snRNP antibodies and antifibrillarin antibodies, respectively, are shown as negative and positive nucleolar staining controls. NHS = normal human serum.

and RNAP I localize to nucleoli (7,8). Thus, in theory, it would make sense to order an anti-RNAP III ELISA after confirming nucleolar staining with immunofluorescent ANAs. However, despite early reports of punctate nucleolar staining by anti-RNAP I antibodies (7,9), other reports suggested that nucleolar staining is not as apparent in sera with anti-RNAP I/III antibodies (2-4,10). One potential explanation is that the punctate nucleolar staining associated with anti-RNAP I antibodies may be obscured by the coexisting nuclear speckled staining of anti-RNAP II and anti-RNAP III antibodies.

In the present study, anti-RNAP I/III antibody-positive sera were examined by double staining HEP-2 ANA slides with an antifibrillarin mouse monoclonal antibody. The specific question of whether nucleolar-positive ANAs can be used as a screening test to order an anti-RNAP III ELISA was evaluated. The relation-

ships between nucleolar staining, levels of anti-RNAP III antibodies versus anti-RNAP I antibodies by immunoprecipitation (IP) assay, and results of anti-RNAP III ELISA were also examined.

PATIENTS AND METHODS

Patients. IP methods were used to identify 13 anti-RNAP I/III antibody-positive sera among samples obtained from 1,119 patients enrolled in the University of Florida Center for Autoimmune Diseases (UFCAD) from 2000 to 2005. Five additional anti-RNAP I/III antibody-positive sera identified in a similar manner were obtained from University of North Carolina (UNC) Hospitals (Chapel Hill, NC). The study protocol was approved by the respective institutional review boards.

IP assay and quantification of anti-RNAP III auto-antibodies. ^{35}S -labeled K562 cell extracts from 2×10^6 cells were immunoprecipitated with serially diluted standard serum

or with 1 μ l of anti-RNAP I/III antibody-positive sera (11). A high-titer standard serum was serially diluted (1:5), starting from a 1:100 dilution, so that 500 μ l of each dilution contained 5, 1, 0.2, 0.04, or 0.008 μ l of the serum. Samples were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed using the Storm PhosphorImager (Amersham Biosciences, Pittsburgh, PA). The volume (sum of the [intensity of signal \times number of pixels]) of the largest subunits of RNAP III and RNAP I antibodies on the phosphorimage was calculated using ImageJ software (NIH Image, National Institutes of Health, Bethesda, MD; online at: <http://rsweb.nih.gov/ij/>). A standard curve was created using the SoftMax Pro version 4.3 program (Molecular Devices, Sunnyvale, CA), and the volume of each band was converted into units.

Immunofluorescent ANAs. Immunofluorescent ANAs (HEp-2 ANA slides; Inova Diagnostics, San Diego, CA) were tested using a 1:160-diluted human serum and Alexa Fluor 488 goat anti-human IgG (1:250 dilution; Molecular Probes, Eugene, OR). Monoclonal antibodies to fibrillar 72B9 (9) (1:10 culture supernatant) and Alexa Fluor 568 goat anti-mouse IgG2a were used to double stain nucleoli. ANAs in 48 SSc sera (13 of which contained anti-RNAP I/III antibodies) from UFCAD and 5 additional anti-RNAP I/III antibody-positive sera from UNC (total of 18) were read in a blinded manner.

In other experiments, HEp-2 cells cultured on slides were fixed using 4 conditions, as follows: 3% paraformaldehyde/phosphate buffered saline (PBS) (10 minutes at 22°C) followed by Tris buffered saline-Tween 20 (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 5 minutes; 3% paraformaldehyde/PBS (10 minutes) followed by 100% methanol (5 minutes at -20°C); acetone/methanol (3:1) for 5 minutes at 4°C; and 100% methanol for 5 minutes at -20°C.

Anti-RNAP III ELISA. An ELISA was performed using an anti-RNAP III ELISA kit (5) (MBL International, Chicago, IL). A high-titer (1:62,500) anti-RNAP III antibody-positive serum diluted 1:5 serially, starting from 1:100, was run as standard. Units correlate with the titers of antibodies: 1:100 = 625 units, 1:500 = 125 units, 1:2,500 = 25 units, 1:12,500 = 5 units, 1:62,500 = 1 unit, and 1:312,500 = 0.2 units. The optical densities of sera diluted 1:100 were converted into units.

RESULTS

IP assay. Phosphorimages from the IP assay using a serially diluted standard serum or 1 μ l of anti-RNAP-positive sera are shown in Figures 1A and B, respectively. A standard curve was created based on the volume of the largest subunit of RNAP III (Figure 1A) or RNAP I (data not shown). The volumes of the largest subunit of RNAP III (black arrowhead in Figure 1A) or RNAP I (white arrowhead in Figure 1A) bands were converted into units, based on the standard curve.

Immunofluorescence. All 18 anti-RNAP I/III-positive sera showed nuclear speckled/homogeneous staining. Nucleolar staining was classified into 3 groups, as follows: 1) nucleolar staining (+): nucleolar staining was clearly present, stronger than or comparable with

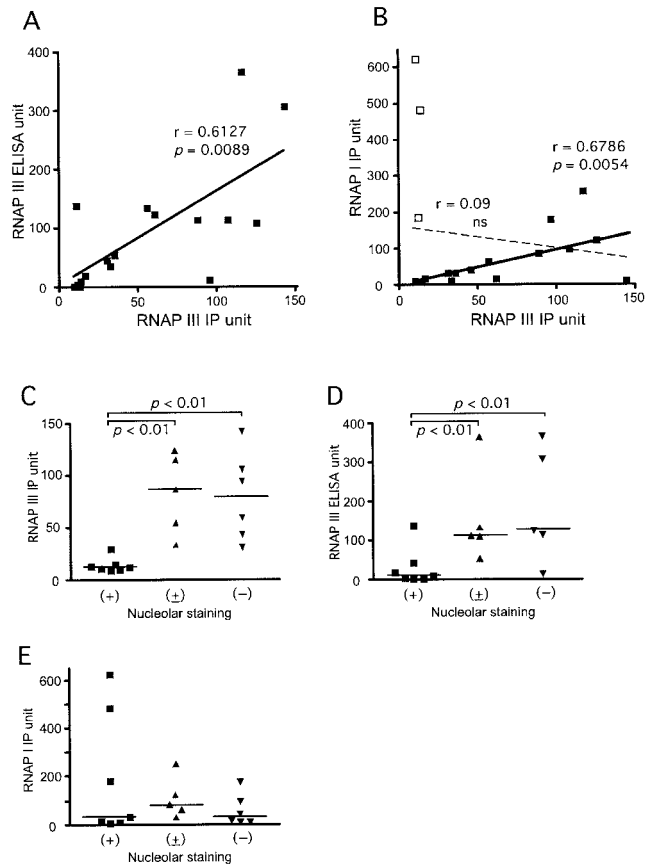


Figure 2. Correlation between the levels of anti-RNA polymerase I/III (anti-RNAP I/III) antibody (as determined by immunoprecipitation [IP] assay), levels of anti-RNAP III antibody (as determined by enzyme-linked immunosorbent assay [ELISA]), and nucleolar staining. **A**, Levels of anti-RNAP III antibodies by ELISA and IP assay had good correlation. **B**, Levels of anti-RNAP I antibody and anti-RNAP III antibody did not correlate if all samples were analyzed (broken line). However, when the 3 sera that predominantly produced anti-RNAP I (open boxes) were excluded, a statistically significant correlation was observed (solid line). ns = not significant. **C**, Levels of anti-RNAP III antibody by IP assay were significantly lower in the group in which nucleolar staining was positive versus the groups in which nucleolar staining was questionable or absent (*P* values determined by Kruskal-Wallis nonparametric test with Dunn’s multiple comparison test). **D**, Levels of anti-RNAP III antibody by ELISA were significantly lower in the group with positive nucleolar staining versus the groups with questionable or negative nucleolar staining (*P* values determined by nonparametric Kruskal-Wallis test with Dunn’s multiple comparison test). **E**, Levels of anti-RNAP I antibody by IP assay were not significantly associated with nucleolar staining; however, some high levels of anti-RNAP I antibody were observed in the group with positive nucleolar staining. Bars in C–E show the means.

nucleolar staining; 2) nucleolar staining (\pm): nucleolar staining appeared to be present but was not obvious and was confirmed by double staining with monoclonal an-

Table 1. Nucleolar staining by sera positive for anti-RNAP I/III, antifibrillar, anti-Th, or anti-nRNP*

Nucleolar staining	Anti-RNAP I/III (n = 18)	Antifibrillar (n = 8)	Anti-Th (n = 3)	Anti-nRNP (n = 7)
Clearly present (+)	42	87	100	0
Not obviously present (\pm)	26	13	0	14
Unrecognizable (-)	32	0	0	86

* Values are the percent. A serum sample with strong anti-U1/U2 RNP and weak anti-U3 RNP is included in both the antifibrillar group and the anti-nuclear RNP (anti-nRNP) group. Anti-RNAP = anti-RNA polymerase. See text for a detailed description of the classification of nucleolar staining.

tibodies to fibrillar; 3) nucleolar staining (-): nucleolar staining was unrecognizable, as confirmed by double staining (Figure 1C).

Some anti-RNAP I/III antibody-positive sera that had strong nucleolar staining were clearly identifiable (Figure 1C, nucleolar +). However, in the majority of cases, nucleolar staining was less clear, and some sera appeared to have weak and limited punctate nucleolar staining, as confirmed by double staining (Figure 1C, nucleolar \pm), while others were clearly negative (Figure 1C, nucleolar -). More than half of the anti-RNAP I/III antibody-positive sera did not show clear nucleolar staining, in sharp contrast to the readily detectable nucleolar staining with all antifibrillar- or anti-Th-positive sera (Table 1). Although the ANA screening was performed in a blinded manner, it was based on readings by experts who had the knowledge that many samples contained anti-RNAP I/III antibodies. This approach may differ from routine practice. Therefore, ANA reports from the local clinical laboratory were reviewed. Data were available for 10 of 13 anti-RNAP I/III antibody-positive sera obtained from UFCAD. All 10 were reported as showing a nuclear speckled pattern without nucleolar staining. In striking contrast, all 9 antifibrillar- or anti-Th-positive sera were reported as having nucleolar staining with or without nuclear staining, except for 1 serum that had antifibrillar and anti-nRNP. These data clearly indicate that nucleolar staining by most anti-RNAP I/III antibody-positive sera was not easily recognizable despite the nucleolar localization of RNAP I.

A potential concern was that the specific slides used in this study might not be optimal for the detection of RNAP I. Thus, 4 commonly used fixation methods for HEP-2 cells were analyzed. All anti-RNAP I/III antibody-positive sera that showed negative nucleolar staining by ANA screening remained negative using any of the 4 fixation methods (data not shown).

Relationship between reactivity by ELISA, IP assay, and immunofluorescence. Levels of anti-RNAP III antibodies as determined by ELISA and by IP assay

correlated positively ($r_s = 0.6127$, $P = 0.0089$) (Figure 2A). A subset of anti-RNAP I/III antibodies recognize the small subunits that are shared between RNAP I and RNAP III autoantibodies (10). However, the levels of anti-RNAP I antibodies and anti-RNAP III antibodies did not correlate well (Figure 2B, thin broken line [P not significant]). This is consistent with the predominant IP of RNAP I (Figure 1B, lanes 1 and 7) or RNAP III (Figure 1B, lanes 2, 9, and 10) by certain sera. When 3 sera that predominantly immunoprecipitated RNAP I were excluded, there was a good correlation between the levels of anti-RNAP I antibody and anti-RNAP III antibody ($r_s = 0.6786$, $P = 0.0054$) (Figure 2B). Unexpectedly, sera with clear nucleolar staining had low levels of anti-RNAP III antibodies, while some sera without nucleolar staining showed high levels of anti-RNAP III antibody ($P < 0.01$ by Kruskal-Wallis nonparametric test and Dunn's multiple comparison test), by IP assay or ELISA (Figures 2C and D). Thus, nucleolar staining appears to have a negative correlation with anti-RNAP III antibodies. In contrast, some nucleolar staining-positive sera showed high levels of anti-RNAP I antibodies (Figure 2E), consistent with its nucleolar localization (7,8).

DISCUSSION

Anti-RNAP III and anti-RNAP I antibodies are almost always present together (2,3) and are often accompanied by anti-RNAP II antibodies (12,13). Nuclear localization of RNAP II was shown previously using monoclonal antibodies (12), but the localization of RNAP I and III was less evident. A recent study using monoclonal antibodies showed that RNAP I localize to nucleoli, whereas RNAP III localizes to nuclei (8).

Early studies (7,9) demonstrated exclusive nucleolar staining by anti-RNAP I antibody-positive sera; however, those results are inconsistent with the findings of more recent studies showing the lack of nucleolar staining by many anti-RNAP I/III antibody-positive sera (2-4,10). The difference is that in the earlier

studies, autoantibodies were examined in SSc sera selected based on predominant or exclusive nucleolar staining (7,9), whereas all SSc sera were screened for anti-RNAP I/III antibodies by IP assay in the later studies (2-4,10). Predominant or exclusive nucleolar staining was observed in 53 of 646 SSc sera, and 7 of these 53 sera had anti-RNAP I/III antibodies as determined by IP assay (7,9). Predominant or exclusive nucleolar staining was observed in only 1.1% of SSc sera (7 of 646) when the sera were selected based on nucleolar staining (9).

This is in striking contrast to a 23% frequency of anti-RNAP I/III using sera from the same institute (University of Pittsburgh) a few years later, when all sera were screened by IP (2). This discrepancy clearly indicates that although some anti-RNAP I/III antibody-positive sera stain nucleoli, predominant or exclusive nucleolar staining is rare. In another study (4), 128 of 735 SSc sera had nucleolar staining with or without a nuclear speckled pattern; 13 of 104 of the former group had anti-RNAP antibodies by IP assay, compared with none of 24 in the latter. Thus, anti-RNAP antibodies were found in only 1.8% (13 of 735) of nucleolar staining-positive SSc sera, in contrast to the actual frequency of 11.7% (86 of 735) (4).

In several studies (2-4,10), all SSc sera were screened by IP assay for anti-RNAP I/III antibodies, and nucleolar staining by the antibody-positive sera was examined. In those studies, the frequency of nucleolar staining was 15-57%, indicating that nucleolar staining by many anti-RNAP I/III antibody-positive sera is difficult to recognize. We originally suspected that this was because of the difficulty in recognizing punctate nucleolar staining (by anti-RNAP I antibodies) coexisting with a nuclear speckled pattern (by anti-RNAP II plus anti-RNAP III antibodies) and tried double staining with antifibrillar monoclonal antibodies as a nucleolar marker. Some sera with inconclusive/suspected nucleolar staining were confirmed to be positive based on this double staining (second group in Table 1). However, nucleolar staining was still unrecognizable in 32% of sera. Thus, it is likely that the high frequency of negative nucleolar staining by anti-RNAP I/III antibody-positive sera observed in previous studies is not related simply to the difficulty in recognizing punctate nucleolar staining. Nevertheless, it should be noted that results of one study suggested that the reactivity with RNAP I large subunits is associated with nucleolar staining (10), and our data also suggest that high levels of anti-RNAP I antibodies are associated with nucleolar staining (Figure 2E). Therefore, reactivity of, and accessibility to, certain

epitopes of RNAP I are associated with nucleolar staining by some, but not all, sera.

There are several explanations for why many anti-RNAP I/III antibody-positive sera are negative for nucleolar staining. First, almost all anti-RNAP I/III antibody-positive sera have a coexistent nuclear speckled pattern by anti-RNAP II and anti-RNAP III antibodies. The bright speckled nuclear staining makes interpretation of nucleolar staining difficult, in that less intense nucleolar staining may be judged negative. Second, the nucleolar staining by anti-RNAP I antibodies is punctate in at least some cases, in contrast to more diffuse (clumpy) nucleolar staining by antifibrillar or anti-Th antibodies (7,9). Punctate nucleolar staining next to a nuclear speckled pattern is difficult to distinguish. For such sera, double staining with another antinucleolar antibody may prove useful, as illustrated in Figure 1D. Third, fixation of cells may denature proteins, destroy some epitopes, and limit accessibility of antibodies to their epitopes. The possibility that subsets/components of RNAP I antibody are more susceptible to extraction, as reported for proliferating cell nuclear antigen (14), also cannot be ruled out. However, none of the 4 fixation methods we used was helpful for detecting nucleolar staining.

Results from the current study as well as those from previous studies clearly indicate that many sera positive for anti-RNAP I/III antibodies do not show apparent nucleolar staining despite the nucleolar localization of RNAP I. Thus, nucleolar staining cannot be used to make decisions regarding whether to order an anti-RNAP III ELISA. It may be reasonable to recommend testing for anti-RNAP III antibodies by ELISA for all patients with SSc, regardless of their ANA staining pattern. Based on the rare coexistence of more than 1 scleroderma-related autoantibody per patient (2), it may be reasonable to exclude patients with anticentromere antibodies or anti-topoisomerase I antibodies from this recommendation.

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