Identification of human autoantibodies to transcription factor IIB

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ABSTRACT

We have characterized the ability of various human autoimmune sera to react with RNA polymerase II transcription factors. One serum, which strongly inhibited transcription in a cell-free system, was shown to contain antibodies directed against human TFIIB. The serum did not show reactivity against the other general transcription factors, including human TBP, TFIIE and TFIIIF. The inhibition of transcription was directly attributable to depletion of TFIIB activity, as demonstrated by reconstitution of activity with recombinant TFIIB. It has long been recognized that components of the RNA processing machinery are major human autoantigens. The present results show that at least one general transcription factor required for messenger RNA synthesis is an autoantigen as well.

INTRODUCTION

Human autoantibodies have been described that react with a variety of nuclear components, including nucleic acids, proteins and protein-nucleic acid complexes (reviewed in 1,2). These antibodies are particularly common in patients with the autoimmune disorders scleroderma, systemic lupus erythematosus, mixed connective tissue disease and Sjögren's syndrome (1,2). Since these antibodies tend to occur in high titers and are often highly specific for the native forms of proteins or protein-nucleic acid complexes, they have proved to be useful for biochemical studies. For example, depletion experiments using autoantisera directed against snRNPs (small nuclear ribonucleoprotein particles) provided the first direct biochemical evidence for a role of ribonucleoprotein particles in mRNA splicing (3,4).

In the present studies, we sought to identify autoantibodies that bind factors involved in RNAP (RNA polymerase) II transcription. Like RNA splicing, transcription is one of the principal metabolic activities of the eukaryotic nucleus and is dependent on the precise assembly of large, multicomponent complexes. Autoantibodies to RNAP I, II and III are relatively common (5-10). Although less common, several examples of autoantibodies specific for RNAP I and RNAP III accessory factors have been reported, including

anti-NOR 90 antibodies, which react with the RNAP I factor hUBF (11,12), antibodies reactive with proteins related to TFIIEA (13), and anti-La antibodies, which react with a factor required for accurate RNAP III transcription (14,15). To our knowledge, however, there are no reports of autoantibodies to RNAP II accessory factors, nor have there been systematic efforts to identify such antibodies.

Two approaches were used to screen human sera for antibodies to RNAP II transcription factors. One was to synthesize the well-characterized basal transcription factors TBP, TFIIB, TFIIE and TFIIIF by coupled *in vitro* transcription-translation and test for immunoprecipitation. A second approach, used with a limited number of sera, was to immunodeplete HeLa cell nuclear extracts and test for residual ability to direct *in vitro* transcription. A total of 132 sera from patients with various autoimmune diseases were screened using one or both approaches. A single positive serum was identified, which both immunoprecipitated *in vitro* translated TFIIB and depleted transcriptional activity from a nuclear extract. Activity was restored to control levels by addition of purified, recombinant TFIIB.

MATERIALS AND METHODS

Sources of autoimmune sera

Samples were selected from serum banks at Keio University School of Medicine, Yale University School of Medicine, and the University of Pittsburgh School of Medicine. All sera used in the study had previously tested positive for antinuclear antibodies. The sample population included 44 patients with systemic lupus erythematosus, 33 patients with scleroderma, 18 patients with various overlap syndromes, and 37 patients whose diagnosis was not known. The panel included 13 sera previously shown to contain antibodies directed against RNAP II (7).

Preparation of radiolabeled proteins by *in vitro* transcription-translation

Radiolabeled human Ku protein and transcription factors were prepared by coupled *in vitro* transcription/translation using a commercial kit (Promega). The cDNA expression plasmids were as follows: TBP (16), subcloned into a T7 expression vector by

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B. C. Hoopes, J. F. LeBlanc and D. K. Hawley (unpublished); TFIIB (17); 34 and 56 kDa subunits of TFIIE (18); 30 and 74 kDa subunits of TFIIF (19,20); and 70 and 80 kDa subunits of Ku autoantigen (21,22). Reactions contained 0.25 μ g of the appropriate expression plasmid DNA, added to a mixture of T7 RNA polymerase, amino acids, [35 S]methionine, RNasin, reaction buffer and nuclease-treated rabbit reticulocyte lysate. Reactions were incubated for 30 min at 30°C. *In vitro* translated proteins were analyzed before use by 12% SDS-PAGE and autoradiography.

Immunoprecipitation

Patient serum was incubated with Protein A-Sepharose or Protein G-Sepharose (Sigma) (1–10 μ l serum to 50 μ l Sepharose) in 500 μ l IPP buffer (10 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1% Nonidet P-40) with rotation for 1 h at 4°C, then washed two to three times with 1 ml of IPP buffer. The antibody-coated Sepharose beads were combined with a mixture of 35 S-radio-labeled transcription factors (3 μ l of each protein solution prepared by *in vitro* transcription/translation) and NET-2 buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Nonidet P-40) in a final volume of 800 μ l. After rotation for 1.5 h at 4°C, the beads were washed three times with 1 ml of NET-2 buffer. During the second and third washes, the beads were incubated for a minimum of 15 min. Samples were transferred to new tubes, centrifuged, and the supernatant discarded. Bound proteins were analyzed by 10% SDS-PAGE and visualized by autoradiography.

RNAP II transcription assay

In vitro run-off transcription assays were performed in a final volume of 50 μ l. Reactions contained 45 ng of a *Hind*III-*Eco*RI fragment from pG5E4T (23), 50 pmol GAL4(1-147) transcriptional activator (24) prepared as described (25), 4 μ l HeLa cell nuclear extract (26), 50 mM KCl, 25 mM Tris-HCl, pH 7.9, 6.25 mM MgCl₂, 0.5 mM EDTA, 5 μ M ZnOAc, 10% glycerol and 10 mM β -mercaptoethanol. Reactions were incubated for 30 min at 30°C, transcription was initiated by addition of nucleotides to a final concentration of 250 μ M ATP, 250 μ M UTP, 250 μ M GTP and 12.5 μ M [α - 32 P]CTP (16 Ci/mmol), and incubation was continued for 60 min at 30°C. Reactions were terminated and samples were analyzed as described (27). Promoter-specific transcription was quantitated by Molecular Dynamics Phosphor-Imager analysis.

Preparation of nuclear extracts depleted with human sera

Protein A-Sepharose beads (Sigma) were washed with TZ 0.1 M buffer (100 mM KCl, 50 mM Tris-HCl pH 7.9, 12.5 mM MgCl₂, 1 mM EDTA, 10 μ M ZnOAc, 20% glycerol, 20 mM β -mercaptoethanol), and 200 μ l of the washed beads and 180 μ l of serum were rotated for 90 min at 4°C. The beads were washed five times with 1 ml TZ 0.1 M buffer. Approximately 200 μ l of supernatant buffer was allowed to remain with the beads. HeLa cell nuclear extract (1 ml) was added and the mixture was rotated for 1 h at 4°C. Beads were centrifuged, and the supernatant was collected, aliquoted, and stored at -80°C.

Expression and purification of recombinant TFIIB

TFIIB was expressed under T7 RNA polymerase control in *Escherichia coli* (6). Two cultures of 1 l each were grown to an

Figure 1. Synthesis of [35 S]methionine-labeled transcription factors. Coupled *in vitro* transcription-translation was performed as described in the Materials and Methods, and samples of the indicated factors were analyzed by 12% SDS-PAGE. Molecular weight markers are indicated on the right (kDa).

OD₆₀₀ of 0.6, induced with 0.4 mM IPTG, incubated for 2 h at 37°C, and the cells collected by centrifugation. Cells were frozen and thawed twice, resuspended in 20 ml ice-cold TM 0.5 M KCl buffer (50 mM Tris, pH 7.9, 12.5 mM MgCl₂, 1 mM EDTA, 1 mM β -mercaptoethanol, 20% glycerol, 0.5 M KCl, 1 μ g/ml PMSF), incubated with 0.2 mg/ml lysozyme for 10 min at room temperature, and sonicated. The lysate was cleared by centrifugation in a Dupont SS-34 rotor at 15 000 r.p.m. for 45 min, and the supernatant was dialysed against TM 0.1 M KCl buffer at 4°C. The dialysed lysate was loaded onto an 80 ml phosphocellulose column, and the column was washed with 160 ml of TM 0.1 M KCl buffer. The column was eluted with a linear gradient of 200 ml TM buffer containing 0.1–1.0 M KCl, at 1 ml/min. Fractions containing TFIIB were identified by SDS-PAGE, pooled, dialysed against TM 0.1 M KCl and concentrated by ultrafiltration. This material was fractionated on a Superdex 75 HiLoad 16/60 column (Pharmacia) equilibrated with TM 0.1 M KCl. The TFIIB was purified to near-homogeneity, as assessed by SDS-PAGE. After identification by SDS-PAGE, fractions containing TFIIB were pooled, aliquoted, and frozen at -80°C.

RESULTS

In order to test human antisera for reactivity with RNAP II transcription factors, radiolabeled TBP, TFIIB, TFIIE (34 and 56 kDa subunits) and TFIIF (RAP30 and RAP74 subunits) were synthesized individually by coupled *in vitro* transcription-translation. In addition to the transcription factors, the 70 and 80 kDa subunits of the Ku protein, a common human autoantigen, were synthesized for use as positive controls. Each reaction produced a major band of the expected size, sometimes contaminated with shorter products that may reflect internal initiation or premature termination (Fig. 1).

The separately translated transcription factors were mixed to facilitate initial screening of sera. Only one of the 132 sera tested gave specific immunoprecipitation of an RNAP II transcription factor. This serum, YM, was from a patient with systemic lupus

Figure 2. Immunoprecipitation of [35 S]methionine-labeled transcription factors using antibodies from human sera. (A) The *in vitro* translated transcription factors shown in Figure 1 were mixed and incubated with Protein A-Sepharose to which human IgG had been adsorbed, as described in the Materials and Methods. Precipitates were analyzed by 10% SDS-PAGE. TB, YM and KK are autoimmune sera, NHS is normal human serum. (B) Immunoprecipitations performed as in (A) except that precipitation of individual transcription factors was assayed separately. Precipitations were performed with YM serum or NHS as indicated. (C) *In vitro* translated TBP was incubated with Protein A-Sepharose to which human serum or purified IgG had been adsorbed, and immune complexes were precipitated and analyzed as in (A). Molecular weight markers are indicated in kDa in all panels.

erythematosus. Representative experiments with YM serum are shown in Figure 2. The YM serum selectively precipitated a 35 kDa polypeptide (Fig. 2A), which corresponds to TFIIB. Several smaller polypeptides, which appear to be incomplete TFIIB translation products, were also precipitated. Two other autoimmune sera used in this experiment, TB and KK, selectively precipitated the 80 kDa subunit of the Ku protein, but did not react with TFIIB.

The specificity of YM serum for TFIIB was confirmed by performing separate immunoprecipitations with individual transcription factors (Fig. 2B). TFIIB was efficiently precipitated. A small amount of TBP was also precipitated. The TBP precipitation appears to be non-specific, as confirmed in Figure 2C, which shows a direct comparison of YM IgG, normal IgG and YM serum, all giving the same low level of TBP reactivity. This weak precipitation was also seen with Protein G-Sepharose beads alone (data not shown).

To confirm that YM antibodies were specific for epitopes in TFIIB itself, rather than an unknown TFIIB-binding protein in the reticulocyte lysate, we performed competition experiments with purified, recombinant TFIIB expressed in *E. coli*. Recombinant TFIIB effectively competes for binding both when added to the immunoprecipitation reaction (Fig. 3, lanes 3 and 4) or when preincubated with the antibody-coated beads (lanes 5 and 6).

We also characterized the YM serum by measuring its ability to deplete transcriptional activity from a HeLa cell nuclear extract. The test promoter, G₅E4T, has five tandem recognition sites for the yeast transcription factor GAL4 located upstream of the adenovirus E4 TATA box (28). This construct has been widely used for studies of activated transcription. It has a low level of basal transcription, which is activated >10-fold by bacterially expressed GAL4(1–147) protein. The GAL4(1–147) protein contains the GAL4 DNA binding domain and an adjacent activation domain (24).

For depletion experiments, IgG from YM and several other human sera were adsorbed to Protein A-Sepharose beads, which were washed and incubated with HeLa cell nuclear extract. The depleted extracts were tested for their ability to direct *in vitro*

Figure 3. Immunoprecipitation of [35 S]methionine-labeled TFIIB can be competed by nonradiolabeled, recombinant TFIIB. Immunoprecipitation was performed as described in Figure 2, except that the concentration of NaCl in NET-2 buffer was increased to 0.5 M at all stages but the final wash. Precipitations used 1 μ l of NHS or YM serum as indicated. In lanes 3 and 4, the indicated amount (μ g) of purified, recombinant TFIIB was added simultaneously with [35 S]methionine-labeled TFIIB. In lanes 5 and 6, antibody-coated Sepharose beads were incubated with purified recombinant TFIIB for 1.5 h at 4°C and washed three times with NET-2 containing 0.5 M NaCl prior to incubation with [35 S]methionine-labeled TFIIB.

transcription, as shown in Figure 4. Antibodies from YM serum decreased transcriptional activity to ~20% of control levels. None of the other sera tested significantly decreased transcription, despite the presence of antibodies to other common human autoantigens (Fig. 4, see legend).

Figure 4. Transcription assays using HeLa cell nuclear extracts depleted with IgG from human sera. Transcription was measured as described in the Materials and Methods, in the absence (solid bars) or the presence (stippled bars) of 50 pmol of GAL4 (1–147). Radiolabeled products were analyzed by 5% urea-PAGE, and incorporation of radiolabel (arbitrary units) into the correctly initiated transcript was quantitated using a Molecular Dynamics PhosphorImager. (A and B) represent separate experiments. In addition to YM serum, the following human sera were used: NHS (normal human serum), HK (anti-Ro/SS-A), FS (anti-Sm), KF (anti-Jo-1), ME (anti-La/SS-B), HT (anti-Ku, anti-Ro), NT (anti-Ku, anti-U1-snRNP), TK (anti-Ku, anti-p350, anti-Ro, anti-Ki).

Figure 5. Transcriptional activity is restored to depleted serum by recombinant TFIIB. Reactions were performed using G₅E4T DNA, GAL4(1–147), and undepleted or YM-depleted HeLa cell nuclear extract, as described in the Materials and Methods. Reactions were supplemented with purified recombinant TFIIB as indicated (ng/reaction). Transcripts were analyzed by 5% urea-PAGE. Size markers are indicated in nucleotides. Arrow indicates 285 nt selective run-off transcript.

To determine whether the observed depletion of transcriptional activity by YM serum was directly attributable to anti-TFIIB reactivity, we reconstituted depleted transcription reactions with purified recombinant TFIIB. Addition of TFIIB restored activity to the depleted extract (Fig. 5). When saturating amounts of TFIIB were present (10–20 ng), the levels of transcription in the depleted and undepleted extracts became equal. Thus, the loss of transcriptional activity in depleted extract is specifically attributable to the loss of functional TFIIB.

DISCUSSION

We have identified a human autoantiserum that depletes an activity that is essential for RNAP II transcription from a HeLa cell nuclear extract. Immunoprecipitation and reconstitution experiments show that the loss of activity results from reaction of the antibodies with TFIIB. To our knowledge, this is the first report of autoantibodies that react with an RNAP II general transcription factor.

There have been several previous reports of autoantibodies to RNAP II itself (7–10). Initially, these antibodies were reported to have a distinct clinical association with systemic sclerosis and to occur in association with antibodies to RNA polymerases I and III (7,8). Recent reports suggest that anti-RNAP II antibodies are more broadly distributed (9,10). Many of the newly identified sera are specific for the elongating form of RNAP II containing a phosphorylated large subunit C-terminal domain (9).

The YM serum did not appear to contain antibodies against RNAP II, as evidenced by the finding that full activity could be restored to a depleted extract by addition of recombinant TFIIB (Fig. 5) and by the absence of RNAP II polypeptides in preliminary immunoprecipitation experiments with ³⁵S-labeled HeLa cell extracts (29). Conversely, none of the 13 anti-RNAP II sera tested in our screening reacted with general transcription factors. Thus, there seems to be no linkage between the presence of antibodies to RNAP II and other components of the transcription apparatus. However, it should be noted that YM contained additional antibodies to U1 RNP, the catalytic subunit of the DNA-dependent protein kinase, and several other, unidentified proteins (29). There is currently no hypothesis to explain this particular antibody cluster.

Most of the major constituents of the eukaryotic nucleus have proved to be recognized to some degree by sera from patients with autoimmune disease. Examples include histones, HMG proteins, nuclear lamins, nucleoplasmin, ubiquitin, topoisomerase I, nucleo-

lar components and various ribonucleoprotein particles, including the major components of the mRNA splicing machinery (reviewed in 1,2). Given that general transcription factors play a prominent and ubiquitous role in the eukaryotic nucleus, it was somewhat surprising that more examples of antibodies to basal transcription factors were not found in the present analysis. It may be that antibodies against general transcription factors are associated with a specific disease type that was not well represented in our panel of sera. Alternatively, the basal transcription factors may participate with a high degree of effectiveness in systems which establish immune tolerance.

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