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Autoantibodies Define a Family of Proteins with Conserved Double-stranded RNA-binding Domains as Well as DNA Binding Activity*

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Cellular responses to viral infection are signaled by double-stranded (ds) RNA, which is not found in substantial amounts in uninfected cells. Although cellular dsRNA-binding proteins have been described, their characterization is incomplete. We show that dsRNA-binding proteins are prominent autoantigens. Sera from B6 and B10.S mice with pristane-induced lupus and human autoimmune sera immunoprecipitated a novel set of 130-, 110-, 90-, 80-, and 45-kDa proteins. The proteins were all major cellular poly(IC)-binding factors. N-terminal amino acid sequences of p110 and p90 were identical and matched nuclear factor (NF) 90 and M phase phosphoprotein 4. p45 and p90 were identified as the NF45·NF90 complex, which binds the interleukin-2 promoter as well as certain highly structured viral RNAs. NF90·NF45 and M phase phosphoprotein 4 belong to a large group of proteins with conserved dsRNA-binding motifs. Besides binding dsRNA, NF90·NF45, p110, and p130 had single-stranded and dsDNA binding activity. Some sera contained autoantibodies whose binding was inhibited by poly(IC) but not single-stranded DNA or *vice versa*, suggesting that the DNA- and RNA-binding sites are different. These autoantibodies will be useful probes of the function of dsRNA-binding proteins. Their interaction with dsRNA, an immunological adjuvant, also could promote autoimmunity.

The existence of viral infection is signaled by the production of double-stranded (ds)[1] RNA, which is not found in substantial amounts in uninfected cells (1). The presence of dsRNA induces interferon production and the expression of a dsRNA-specific adenosine deaminase (2, 3) and the dsRNA-dependent serine/threonine kinase PKR (4). PKR phosphorylates eukaryotic protein synthesis initiation factor 2 and IκB, resulting in a global inhibition of protein synthesis and activation of NFκB, respectively (5, 6). It also activates interferon regulatory factor 1 (7).

The cellular machinery for recognition of dsRNA has come under increasingly close scrutiny. Structured viral RNAs, such as the adenovirus virus-associated (VA) RNA_{II}, are recognized by cellular dsRNA-binding proteins such as RNA helicase A, the NF90·NF45 dimer, and other less well defined factors (8). The components of the dsRNA recognition apparatus and their functions are poorly characterized, although some of them carry one or more copies of a conserved dsRNA-binding motif (9). Interestingly, although the NF90 protein carries two copies of this motif, it also binds to a DNA element found in the interleukin-2 promoter (10, 11). However, the significance of this dual nucleic acid binding activity is not known.

Sera from patients or mice with systemic autoimmune diseases have been instrumental in defining the function of the proteins associated with small nuclear ribonucleoprotein particles (12). One of these, the La (SS-B) autoantigen has dsRNA binding activity (13), but autoantibodies against the major dsRNA-binding proteins that bind to VA RNA_{II} have not been reported. In the present study autoantibodies specific for a family of dsRNA-binding factors, including those implicated in VA RNA_{II} binding, were identified. Interactions between these factors were characterized along with their nucleic acid binding properties.

EXPERIMENTAL PROCEDURES

Treatment of Mice—Four-week-old female SJL/J, A.SW, B10.S, C57BL/10 (B10), BALB/c ByJ, BALB.B, BALB.K, C57BL/6J (B6), and B6H2k mice were purchased from Jackson Laboratory (Bar Harbor, ME) and housed conventionally. Additional B6 mice were purchased from Charles River Laboratories (Wilmington, MA). At 10 weeks of age, 5–20 mice/strain were injected with 0.5 ml of pristane intraperitoneal or with an equal volume of sterile phosphate-buffered saline (PBS) as a control. Sera were collected from the tail vein before injection, at 2 and 4 weeks afterward and at 1-month intervals thereafter. Sera from DBA/1 mice 6 months after pristane treatment were a gift of Dr. Paul H. Wooley (Wayne State University, Detroit, MI).

Radiolabeling and Immunoprecipitation—Autoantibodies to cellular proteins in murine and human sera were analyzed by immunoprecipitation of ^{35}S-radiolabeled K562 cell extract using 3 μl of murine serum/sample as described (14). Specificity was confirmed using human anti-nRNP/Sm, Su, and ribosomal P reference sera.

Immunoblotting—IgG from 10 μl of human serum YM was cross-linked to 50 μl (v/v) of protein A-Sepharose beads with dimethyl pimelimidate as described (15). K562 cells were sonicated in NET/Nonidet P-40 (150 mM NaCl, 2 mM EDTA, 50 mM Tris, pH 7.5, 0.5% Nonidet P-40) at a concentration of 4 × 10^7/ml and cleared by centrifugation. Cell extract from 10^8 cells was incubated with patient YM IgG-protein A-Sepharose beads for 1 h at 4 °C. The p130, p110, p90, and p45 antigens were affinity-purified, fractionated by SDS-PAGE, and transferred to nitrocellulose. Strips were probed with sera from pristane or PBS-treated mice, human autoimmune sera, or normal human serum (1:2000 dilution). They then were incubated with 1:3000 alkaline phosphatase-conjugated goat anti-mouse IgG or goat anti-human IgG (γ-chain-specific, Southern Biotechnology, Birmingham, AL) and devel-

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[1] The abbreviations used are: ds, double-stranded; NF, nuclear factor; ss, single-stranded; VA, virus-associated; B6, C57BL/6 mice; MPP4, M phase phosphoprotein 4; PKR, dsRNA-dependent protein kinase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

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oped using the Western-Star chemiluminescent system (Tropix, Bedford, MA).

To verify the identities of the antigens, proteins immunoprecipitated by human sera YM and EB were fractionated by SDS-PAGE, transferred to nitrocellulose, and probed with serum from a pristane-treated B6 mouse containing antibodies to p110, p90, and p45 or with PBS-treated mouse serum. In addition, strips were probed with serial B6 or B10.S sera obtained 1–6 months after pristane treatment to examine the onset of autoantibody production.

Affinity Purification of Autoantibodies—The p130, p110, p90, and p45 proteins were affinity-purified from K562 cell extract using human autoimmune serum, fractionated by SDS-PAGE, and transferred to a nitrocellulose filter as above. After Ponceau red staining, the p130, p110, p90, and p45 bands and unrelated areas were cut from the filter. Strips were incubated with 2 ml of 1:1000 B6 or B10.S sera for 1 h at 22 °C and washed with TBS-Tween 20 (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20). Bound antibodies were eluted twice with 300 μl of 50 mM glycine, 150 mM NaCl, pH 2.9. After adjusting the pH to 7.5 with 0.2 M Na_2HPO_4, affinity-purified antibodies were used to reprobe strips with bound p130, p110, p90, and p45.

N-terminal Amino Acid Sequence—The p130, p110, p90, and p45 proteins from K562 cell extract were affinity-purified with human serum bound to protein A-Sepharose beads (see above), fractionated by SDS-PAGE, and transferred to polyvinylidene difluoride membrane. The membrane was stained with 0.1% Coomassie Blue in 40% methanol and destained with 50% methanol, and the proteins were subjected to N-terminal amino acid sequencing using a Perkin-Elmer/ABI Procise model 492 protein/peptide sequencer (University of North Carolina/ Program in Molecular Biology and Biotechnology Micro Protein Chemistry Facility). Proteins exhibiting homology with the N-terminal sequences were identified using the NCBI advanced BLAST algorithm for the GenBank™ Data Base.

Enzyme-linked Immunosorbent Assays for Autoantibodies to NF90·NF45—Full-length recombinant NF45 and NF90 with N-terminal 6-histidine tags were expressed in *Escherichia coli* and purified by nickel chelate affinity column as described (10). The wells of microtiter plates (MaxiSorp, Nunc, Naperville, IL) were coated with 50 μl of 2 μg/ml of purified recombinant NF45 or NF90 in 20 mM Tris-HCl, pH 8.0, for 16 h at 4 °C. Wells were washed with NET/Nonidet P-40 and blocked with 0.5% bovine serum albumin in NET/Nonidet P-40 for 1 h at 22 °C followed by incubation with 100 μl of 1:500 mouse serum in blocking buffer for 2 h. Wells were washed with NET/Nonidet P-40, incubated with 100 μl of 1:1000 alkaline phosphatase-conjugated goat anti-mouse IgG for 2 h, and developed.

Poly(IC) and DNA Affinity Chromatography—120 μl (50% v/v) of poly(IC)-agarose (Amersham Pharmacia Biotech), ssDNA cellulose, or dsDNA cellulose (Sigma) in TBS-Tween 20 plus 3 mM $MgCl_2$ was washed three times with starting buffer. The matrix was incubated for 1 h at 4 °C with cell extract from 10^7 [^{35}S]methionine-labeled K562 cells in starting buffer plus phenylmethylsulfonyl fluoride and aprotinin and then washed six times with 1 ml TBS-Tween 20 + 3 mM $MgCl_2$. Proteins were eluted with 600 μl of 1.5 M NaCl NET/Nonidet P-40 for 20 min at 4 °C. The eluate was spun three times in a microcentrifuge for 5 s, and the supernatant was transferred to a new tube and adjusted to a final concentration 0.75 M NaCl. The eluate was immunoprecipitated (30 min at 4 °C) with 5 μl of human autoimmune serum YM containing anti-NF45·NF90, anti-Ku + nRNP/Sm, or normal human serum, washed three times with 0.5 M NaCl NET/Nonidet P-40, and washed once with NET before analyzing by SDS-PAGE.

Inhibition Assay—A microtiter plate was coated with 50 μl of 0.5 μg/ml recombinant NF90 or NF45 at 4 °C for 14 h. Wells were washed twice with TBS-Tween 20 plus 3 mM $MgCl_2$ and blocked for 1 h with 0.5% bovine serum albumin in the same buffer. Wells coated with NF90 or NF45 were incubated for 3 h at 22 °C with single-stranded (ss) DNA (from calf thymus, Sigma) or poly(IC) (Sigma) at a concentration of 10, 1, 0.1, 0.01, and 0.001 μg/ml in PBS + 3 mM $MgCl_2$ or with buffer alone. Wells were washed three times with TBS-Tween 20 + 3 mM $MgCl_2$, incubated with murine or human autoimmune sera (1:500–1:10,000) in 0.5% bovine serum albumin TBS-Tween 20, 3 mM $MgCl_2$, and washed three times with TBS-Tween 20. Wells were incubated for 1 h at 22 °C with 100 μl of alkaline phosphatase-conjugated goat anti-human or anti-mouse IgG antibodies (γ-chain-specific, 1:1000) and developed using Sigma 104 substrate. Inhibition was calculated as follows: [(A_{405} of wells incubated with buffer alone − A_{405} of wells incubated with ssDNA or poly(IC)) ÷ A_{405} of wells incubated with buffer alone] × 100.

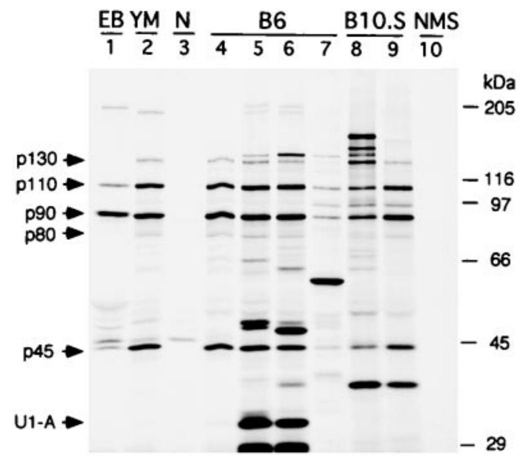

FIG. 1. **Autoimmune sera immunoprecipitate p130, p110, p90, p80, and p45.** ^{35}S-Labeled K562 cell extract was immunoprecipitated with sera from patients with systemic lupus erythematosus-rheumatoid arthritis overlap syndrome (patient *EB*, *lane 1*) or scleroderma (patient *YM*, *lane 2*) or with normal human serum (*N*, *lane 3*). The same extract was also immunoprecipitated with B6 sera (*lanes 4–7*), B10.S sera (*lanes 8* and *9*) 6 months after pristane treatment or with control mouse serum (*NMS*, *lane 10*). All sera immunoprecipitated p130, p110, p90, p80, and p45 except for the EB (*lane 1*), which did not immunoprecipitate p130 or p80 clearly but did immunoprecipitate the other proteins.

RESULTS

Sera from mice treated with pristane contain autoantibodies against antigens recognized by human systemic lupus erythematosus sera (14, 16). Some are RNA-associated proteins, such as the components of U1 small nuclear ribonucleoproteins. In the present study, we investigated a novel set of proteins recognized by sera from pristane-treated mice and certain human autoimmune sera. These proteins included some of the major proteins bound by VA RNA_{II}, indicating that dsRNA-binding proteins are a novel group of autoantigens.

Autoantibodies to p130, p110, p90, p80, and p45—When sera from various strains of pristane-treated mice were tested at 6 months, some B6 (Fig. 1, *lanes 4–7*) and B10.S (*lanes 8* and *9*) mouse sera immunoprecipitated a set of 130-, 110-, 90-, 80-, and 45-kDa proteins. Normal mouse serum (*lane 10*) did not. Out of 31 B6 mice treated with pristane, nine (29%) had this specificity. Among these, four also had anti-nRNP/Sm, two had anti-ribosomal P, and one had anti-Su antibodies. Two of six pristane-treated B10.S mice had this specificity, both with co-existing anti-ribosomal P and anti-Su antibodies. These proteins were not immunoprecipitated by sera from A.SW, BALB/c ByJ, or SJL/J mice, despite the high frequencies of anti-nRNP/ Sm, Su, or ribosomal P antibodies reported previously in these strains (14, 16).[2]

Two human autoimmune sera immunoprecipitated a similar set of proteins (Fig. 1, *lanes 1* and *2*), whereas normal human serum (*lane 3*) did not. The serum used in *lane 1* was from EB, an Afro-Caribbean patient with systemic lupus erythematosus-rheumatoid arthritis overlap syndrome, and the serum in *lane 2* was from YM, a Japanese patient with scleroderma. All sera with this specificity immunoprecipitated proteins of ~130, 110, 90, 80, and 45 kDa with the exception of EB (*lane 1*), which did not immunoprecipitate the 130- or 80-kDa polypeptides clearly. The relative intensities of p110, p90, and p45 immunoprecipitated by all sera except EB were comparable. The stronger p90 signal using EB serum was found to be due to the immunoprecipitation of two proteins migrating at ~90 kDa. Using differ-

[2] M. Satoh, H. B. Richards, V. M. Shaheen, H. Yoshida, M. Shaw, P. H. Wooley, and W. H. Reeves, submitted for publication.

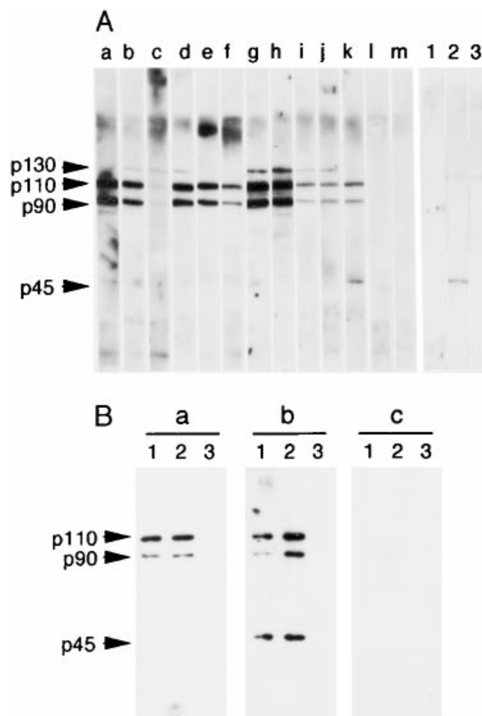

FIG. 2. **Immunoblot analysis of the specificities of human and murine sera.** *A*, p130, p110, p90, and p45 were affinity-purified from K562 cell extract using patient EB IgG-Sepharose, fractionated by SDS-PAGE, and transferred to nitrocellulose. Strips were probed with sera from pristane treated B6 (*lanes a–i*), or B10.S (*lanes j* and *k*) mice or with PBS-treated B6 sera (*lanes l* and *m*). Sera tested in *lanes a–k* immunoprecipitated p130, p110, p90, and p45. Note consistent reactivity with p110 and p90 on the blots. Some sera also were weakly reactive with p130 and/or p45 (p45, see *lanes a* and *k*). Additional strips were probed with patient EB or YM sera (*lanes 1* and *2*) or with normal human serum (*lane 3*). EB serum was unreactive with any proteins on membrane whereas YM serum reacted with p45 weakly. *B*, K562 cell extract was immunoprecipitated with EB and YM sera (*lanes 1* and *2*, respectively) or with normal human serum (*lane 3*). Proteins were fractionated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with sera from a pristane-treated B6 mouse with antibodies to p110, p90, and p45 (*panel a*), from a pristane-treated B10.S mouse with antibodies to p110, p90, and p45 (*panel b*) or from a PBS-treated mouse serum (*panel c*). Mouse sera recognized proteins immunoprecipitated by human autoimmune sera EB and YM.

ent gel conditions, the two ~90-kDa bands immunoprecipitated by EB serum could be resolved (not shown).

Human and Murine Autoimmune Sera Recognize the Same Antigens—The relationship of the proteins immunoprecipitated by sera from patients EB and YM to those recognized by sera from B6 or B10.S mice was investigated by Western blotting. The p130, p110, p90, and p45 proteins were affinity-purified using patient YM IgG-Sepharose, separated by SDS-PAGE, and transferred to nitrocellulose (Fig. 2*A*). Blots were probed with sera from pristane-treated B6 (*lanes a–i*) or B10.S (*lanes j* and *k*) mice or with sera from PBS-treated B6 mice (*lanes l* and *m*). Additional strips were probed with EB and YM sera (*lanes 1* and *2*, respectively), or normal human serum (*lane 3*). All sera from pristane-treated mice that immunoprecipitated p130, p110, p90, p80, and p45 recognized p110 and p90 by Western blot (*lanes a–i*). Many sera also reacted weakly with p130 and some recognized p80 (*lanes a* and *h*) or p45 as well (*lanes a* and *k*). In contrast, the human sera were poorly reactive on immunoblotting. EB serum (Fig. 2*A*, *lane 1*) did not recognize any of the proteins in Western blot, whereas YM serum reacted weakly with p45. However, unlike the murine sera, it did not react with p110 or p90 (Fig. 2*A*, *lane 2*). Thus, human autoantibodies recognized mainly native conformational epitopes.

P110	- - PMRIFVND
P90	- - PMRIFVND
MPP4	MRPMRIFVNDDRHVMAKHSSVY
NF90	MRPMRIFVNDDRHVMAKHSSVY
Xenopus dsRNA bp	MRPMRIFLNDDRHVMAKHSVVY

FIG. 3. **Comparison of N-terminal sequences.** N-terminal amino acid sequences of p110 and p90 were aligned with the N-terminal sequences of MPP4, NF90, and *Xenopus* dsRNA-binding protein obtained from GenBank™.

The identity of the antigens recognized by the human and murine sera was further verified by immunoprecipitating K562 extract with patient EB or YM serum (Fig. 2*B*, *lanes 1* and *2*, respectively) or with normal human serum (*lane 3*) followed by immunoblotting. Blots were probed with sera from a B6 mouse with anti-p110 and p90 (Fig. 2*B*, *panel a*), from a B10.S mouse with anti-p110, p90, and p45 (*panel b*), or from a PBS-treated B6 mouse (*panel c*). Mouse sera with antibodies to p110, p90, and/or p45 recognized proteins of the same size that were immunoprecipitated by human autoimmune sera EB and YM, confirming that the proteins recognized by the human and murine autoimmune sera were the same.

p90 and p45 Are NF90·NF45—To identify the proteins recognized by the human and murine autoimmune sera, N-terminal sequences were obtained from p110 and p90 affinity-purified on patient YM IgG-protein A-Sepharose. The p110 and p90 proteins had identical N-terminal amino acid sequences: PMRIFVND. A homology search identified two proteins with the same N-terminal sequence: M phase phosphoprotein 4 (MPP4) and nuclear factor (NF) 90 (Fig. 3). A homologue of MPP4, *Xenopus* double-stranded (ds) RNA-binding protein, matched the N-terminal sequence of p110 and p90 at 7 of 8 positions. The p90 protein was identified as NF90 on the basis of several criteria: 1) the sizes were similar, 2) N-terminal amino acid sequences were identical, 3) sera from rabbits immunized with NF90 recognized p90 on Western blots (not shown), and 4) autoimmune sera with anti-p90 recognized recombinant NF90 (see Fig. 6).

Although the N-terminal sequence could not be obtained, possibly because of a blocked N terminus, p45 was identified as NF45 on the basis of similar sizes and its physical association with NF90. NF90 and p45 co-migrated on sucrose gradients, suggesting that they are physically associated, and NF90 could be co-immunoprecipitated by affinity-purified anti-p45 antibodies (not shown). Finally, serum from a rabbit immunized with NF45 recognized p45 on immunoblots and the murine autoimmune sera containing anti-p45 antibodies were reactive with recombinant NF45 on Western blots (see Fig. 6). On the basis of the biochemical and immunological data, we conclude that p90 and p45 are NF90 and NF45, respectively.

p110, p130, and p80—The identity of the 110-kDa protein is less certain. Autoantibodies affinity purified from p90 cross-reacted with p110, and serum from a rabbit immunized with NF90 recognized the 90- and 110-kDa proteins immunoprecipitated by the human and murine autoimmune sera (not shown). The 110-kDa protein could be MPP4 (17), which was found to share an identical N-terminal amino acid sequence with NF90 by aligning the sequences using the BLAST programs. The reported partial sequences of MPP4 are nearly identical with NF90. MPP4 is recognized by monoclonal antibodies specific for the phosphorylated sites of proteins (17, 18) and is a nuclear protein phosphorylated in M phase thought to be a human

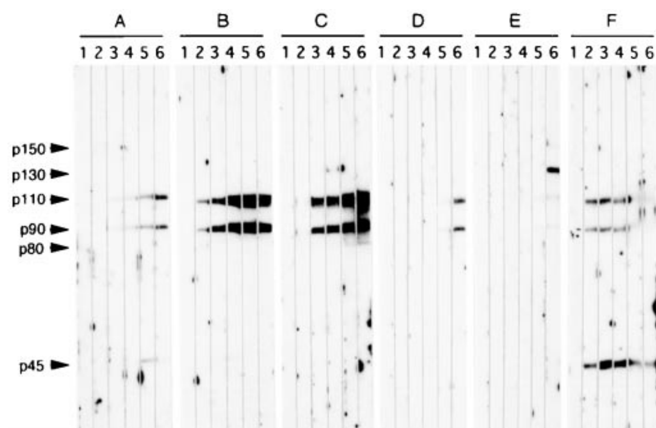

FIG. 4. **Time course of autoantibody production.** The p130, p110, p90, and p45 antigens were affinity-purified from K562 cell extract, fractionated by SDS-PAGE, and transferred to nitrocellulose. Strips were probed with serial sera obtained 1, 2, 3, 4, 5, and 6 months after pristane treatment of B6 mice (*A–D*) or B10.S mice (*E* and *F*). Positions of the p130, p110, p90, and p45 antigens are indicated.

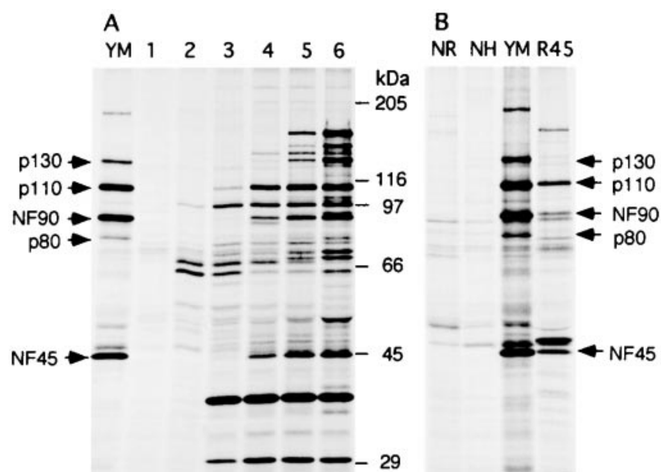

FIG. 5. **NF45 forms complexes with NF90 and p110.** *A*, immunoprecipitation using sera from a pristane-treated B10.S mouse. Sera were collected monthly for 6 months and used to immunoprecipitate proteins from K562 cell extract (*lanes 1–6*). Immunoprecipitation with a human reference serum is shown on the *left*. Positions of p130, p110, NF90, and NF45 are indicated. *B*, immunoprecipitation using rabbit anti-NF45 antiserum. Serum from a rabbit immunized with recombinant NF45 expressed in bacteria immunoprecipitated NF45 as well as NF90 and p110 (*lane RA45*). Immunoprecipitation with human reference serum (*YM*, positive control) is shown for comparison, as well as normal human serum (*NH*) and preimmune rabbit antiserum (*NR*).

homologue of *Xenopus* dsRNA-binding protein. Alternatively, p110 could be an extended form or precursor of NF90 (see "Discussion").

The p130 and p80 polypeptides co-purified with NF45·NF90 on a poly(IC)-agarose column and may be related to NF45·NF90 (see Fig. 7). It is possible that p80 is a degradation product of p130, p110, or NF90. However, p130 and p80 are unlikely to form a stable complex with NF45·NF90 because patient EB serum did not co-immunoprecipitate p130 or p80 (Fig. 1).

Time Course of Autoantibody Production—The data shown in Figs. 1–3 suggested that the production of autoantibodies to p130, p110, p90 (NF90), and p45 (NF45) was tightly linked. For this reason, we investigated the time course of autoantibody production by Western blot in sera obtained sequentially from pristane-treated mice (Fig. 4). Affinity-purified antigens were probed with sera obtained 1–6 months after pristane treatment of B6 (*panels A–D*) or B10.S mice (*panels E* and *F*). Antibodies to p110 and NF90 always developed simultaneously between 2–5 months. Some sera also recognized NF45 (*panels A* and *F*) or p130 (*panel E*). In the B6 mouse illustrated in panel A, antibodies to NF90 and p110 were detected at 3 months, whereas antibodies to NF45 did not appear until 5 months after pristane treatment.

The time course also was investigated by immunoprecipitation using serial sera from these mice. NF90, NF45, and p110 usually appeared in the immunoprecipitates at the same time. However, occasionally p110 and NF45 were immunoprecipitated before immunoprecipitation of NF90 was apparent (Fig. 5*A*). Sera from this B10.S mouse immunoprecipitated p110 plus NF45 at 3 months, followed by an increased amount of NF45 plus NF90 at 4 months. At 5 months, the mouse developed autoantibodies against p130. This suggested that NF45 bound both p110 and NF90, a possibility that was investigated further by using rabbit antiserum against NF45. As shown in Fig. 5*B*, anti-NF45 antiserum (*lane R45*) co-immunoprecipitated both NF90 and p110, further suggesting that NF90·NF45 and p110·NF45 dimers may exist. In contrast, p130 was not co-immunoprecipitated by anti-NF45, suggesting that it did not form a stable complex with NF45. This interpretation also was supported by the fact that p130 could be eluted from poly(IC)-agarose by 0.5 M NaCl, whereas NF90, NF45, and p110 were eluted only at higher salt concentrations (not shown). Because the results of the co-immunoprecipitation studies could be compatible with immunological cross-reactivity between NF45 and other proteins, the specificities of anti-NF45, NF90, and p110 autoantibodies also were examined by Western blot.

Autoantibodies to p110 and NF90 Are Cross-reactive—Sera from a pristane-treated B6 mouse (Fig. 6, *panel a*, *lane 1*) or from a B10.S mouse (*panel b*, *lane 1*) recognized both p110 and NF90 in Western blot assays. Affinity-purified anti-p110 and anti-NF90 from the sera bound to both p110 and NF90 (*lanes 3* and *4*, respectively), suggesting that these autoantibodies were cross-reactive. The signal from affinity-purified anti-NF45 (*panel b*, *lane 5*) was weak under these conditions but apparently specific for NF45. Thus, immunological cross-reactivity is likely to explain the simultaneous onset of anti-NF90 and anti-p110 autoantibodies in many sera. These data also lend further support to the idea that NF45 forms a complex with NF90 and with p110.

NF45·NF90 complexes bind to dsRNA as well as DNA. NF90 has two copies of a dsRNA-binding motif, binds dsRNA strongly, and interacts selectively with the adenoviral VA RNA_{II}, suggesting that it could play a role in regulating the activity of certain highly structured RNAs along with its proposed role as a DNA-binding protein (10, 8). The nucleic acid binding properties of proteins recognized by the human and murine autoimmune sera were examined. Proteins from K562 cell extract were absorbed to dsRNA (poly(IC)-agarose), ssDNA, or dsDNA cellulose, eluted with salt, and immunoprecipitated with autoimmune sera. Immunoprecipitation with human serum YM (anti-NF45·NF90) is shown in *lane 1* of each panel. *Lanes 2* and *3* show immunoprecipitations with human anti-Ku plus anti-nRNP/Sm serum or normal human serum, respectively. NF45·NF90 complexes were detected in eluates from poly(IC)-agarose as reported previously (8), as well as in eluates from ssDNA and dsDNA cellulose (Fig. 7, *lanes 1*).

The Ku (p70·p80) complex, which binds to ends of dsDNA or single to double strand transitions, but not RNA, was used as a specificity control (*lanes 2*). Consistent with previous observations (reviewed in Ref. 19), Ku antigen was detected in eluates from ssDNA and dsDNA cellulose. However, it was not present in the eluates from poly(IC)-agarose. Another control antigen, the U1 small nuclear ribonucleoprotein, could be detected

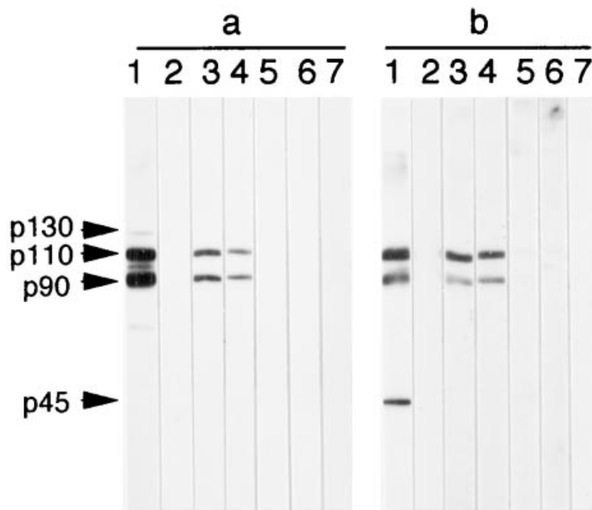

FIG. 6. **Autoantibodies to p110 and p90 are cross-reactive.** The p130, p110, p90, and p45 proteins were affinity-purified, fractionated by SDS-PAGE, and transferred to nitrocellulose. After staining with Ponceau red, bands at 130, 110, 90, and 45 kDa and an unrelated area of the blot were cut out of the membrane. The individual protein bands were incubated with autoimmune serum, and bound antibodies were affinity-purified and used to reprobe strips of the original blot. Affinity-purified antibodies to p110 (*lanes 3*) and p90 (*lanes 4*) bound to both p110 and p90, suggesting that these antibodies are cross-reactive. *Panel a*, serum from a pristane-treated B6 mouse. *Panel b*, serum from a B10.S mouse treated with pristane. Individual lanes were probed with the following: *lane 1*, starting serum (without affinity purification); *lane 2*, antibodies affinity-purified from p130; *lane 3*, antibodies affinity-purified from p110; *lane 4*, antibodies affinity-purified from p90; *lane 5*, antibodies affinity-purified from p45; *lane 6*, antibodies affinity-purified from irrelevant region of the blot; *lane 7*, serum from a PBS-treated mouse.

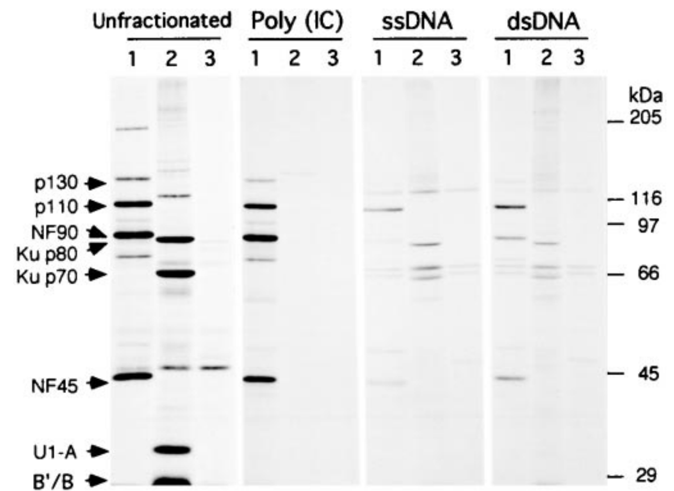

FIG. 7. **NF45·NF90 complexes bind to dsRNA as well as DNA.** [^{35}S]Methionine-labeled K562 cell extract was incubated with poly(IC)-agarose, ssDNA cellulose, or dsDNA cellulose, and bound proteins were eluted with 1.5 M NaCl NET/Nonidet P-40. Whole K562 cell extract or proteins eluted from dsRNA poly(IC)-agarose, ssDNA, or dsDNA cellulose were immunoprecipitated with human sera containing anti-NF45·NF90 (*lanes 1* of each panel), anti-Ku plus anti-nRNP/Sm (*lanes 2*), or with normal human serum (*lanes 3*). Note the presence of NF45·NF90 complexes in eluates from dsRNA, as well as the ssDNA and dsDNA affinity matrices (*lanes 1*). In contrast, Ku70·Ku80 complexes (DNA-binding protein complex) were found only in the eluates from ssDNA and dsDNA affinity matrices (*lanes 2*).

readily in the unfractionated extract (*left panel*, see U1-A protein) but was not present in eluates from any of the three affinity matrices. Thus, binding of the NF45·NF90 complex to dsRNA as well as ssDNA and dsDNA was confirmed. The p130 and p80 proteins also were eluted from poly(IC)-agarose. Although there was evidence that p130 interacts with ssDNA and dsDNA, we cannot be certain whether this protein binds DNA or dsRNA independently or because of an interaction with NF45·NF90.

Binding of Autoantibodies to NF90 Is Inhibited by ssDNA and Poly(IC)—The nucleic acid-binding site of Ku antigen is a target of autoantibodies (20). Because the NF45·NF90 dimer binds to dsRNA as well as DNA, we examined whether nucleic acid binding could block autoantibody recognition. The binding of antibodies to NF90 in two B6 mouse sera (Fig. 8, *A* and *B*) was inhibited by poly(IC) in a dose-dependent manner, but only weakly by ssDNA. The binding of other sera to NF90 was inhibited by both poly(IC) and ssDNA or by ssDNA alone (Figs. 8, *C* and *D*). Neither ssDNA nor poly(IC) inhibited binding to NF45, suggesting that NF45 does not bind nucleic acids by itself or that the nucleic acid-binding site is not recognized by autoantibodies. Of the 11 anti-NF90·NF45 positive sera from pristane-treated mice tested, the binding of four to NF90 was inhibited >10% by ssDNA (Fig. 8*C*, *left*). In contrast, the binding of these sera to NF45 was not inhibited by ssDNA (Fig. 8*C*, *right*). Poly(IC) inhibited the binding of 7/11 sera by >10%. Although both ssDNA and poly(IC) inhibited the binding of many sera, in some cases only one of the two nucleic acids inhibited (Fig. 8*D*). In the case of human autoimmune serum YM, only poly(IC) inhibited binding to NF90. These data suggest that the DNA and dsRNA-binding sites of NF90 are not identical. Moreover, anti-NF90 autoantibodies recognized epitope(s) near the DNA- and dsRNA-binding sites, consistent with the idea that autoantibodies often recognize functional sites (21).

DISCUSSION

Double-stranded RNA is a signature of viral infection, and host cells have evolved defense mechanisms for detecting and responding to it. Several dsRNA-binding proteins, some sharing a conserved dsRNA-binding motif, have been identified (9). In this study, we present evidence that prominent dsRNA-binding proteins of 130, 110, 90 (NF90), 80, and 45 (NF45) kDa are recognized by human and murine autoantibodies. Biochemical studies suggest that at least some these proteins are physically associated and that the 90- and 110-kDa proteins share significant sequence homology and immunological cross-reactivity.

dsRNA-binding Proteins: a Novel Class of Autoantigens—A variety of DNA-protein and RNA-protein antigens are recognized by autoantibodies in systemic autoimmune diseases (12, 22). However, dsRNA-binding proteins have not previously been considered as autoantigens. In the present study, we identified novel autoantibodies against a set of at least five proteins recovered in high salt eluates of poly(IC)-agarose affinity columns (Fig. 7), suggesting that they are dsRNA-binding factors.

The 90- and 45-kDa proteins were shown to be NF90·NF45, a heterodimer initially isolated as a nuclear factor that binds to the AARE element of the human interleukin-2 promoter (11). The DNA binding activity of this factor is augmented by ionomycin plus phorbol 12-myristate 13-acetate and inhibited by cyclosporin or FK506 (10). More recently, NF90·NF45 was found to bind selectively to the adenoviral VA RNA_{II} (8), suggesting that it also binds to dsRNA. Consistent with that possibility, NF90 has sequence similarity at positions 419–464 and 535–604 to the dsRNA-binding site of *Drosophila* Staufen, human PKR, human trans-activation-responsive RNA-binding protein, and other proteins with a conserved dsRNA-binding motif (10). It recently has been shown to exhibit dsRNA binding activity and to be phosphorylated in a dsRNA-dependent manner, possibly by interferon-induced PKR (23). NF90 is highly homologous with MPP4 and *Xenopus* dsRNA-binding protein

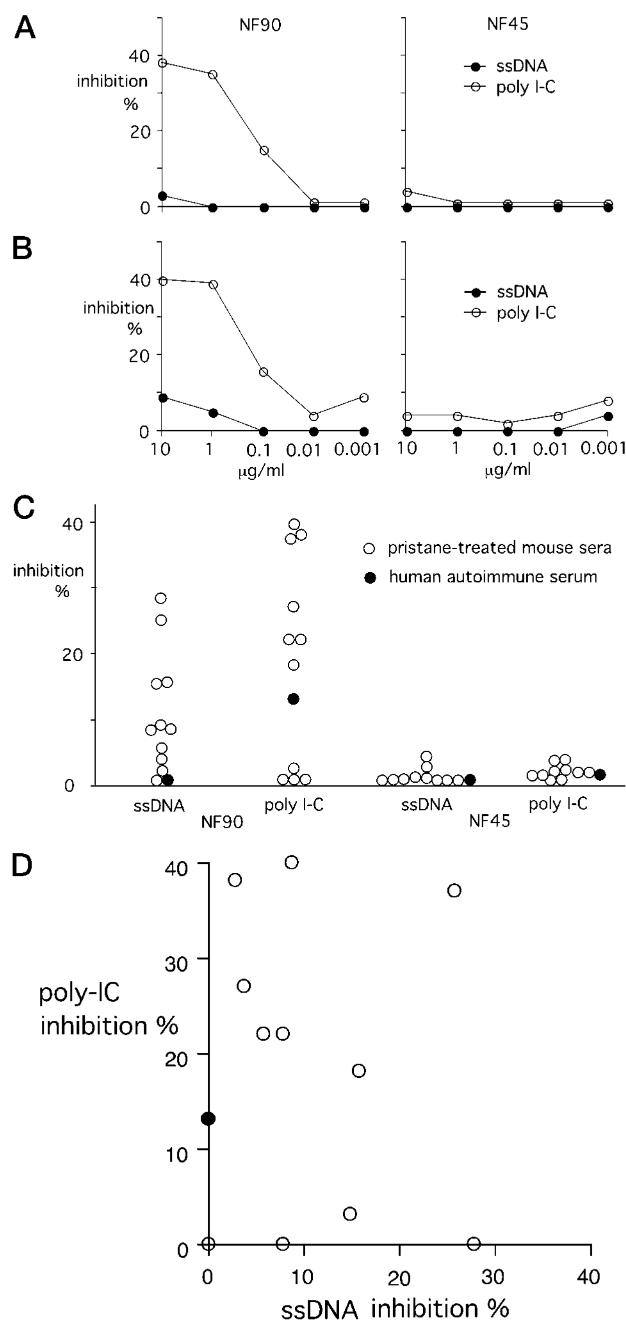

FIG. 8. **DNA and dsRNA inhibit autoantibody binding.** *A* and *B*, microtiter plate wells were coated with recombinant NF90 or NF45 and incubated with ssDNA or poly(IC) at indicated concentrations. Wells were washed and incubated with 1:500 sera from a B6 (*A*) or a B10.S (*B*) mouse and then incubated with 1:1000 alkaline phosphatase-conjugated goat anti-mouse IgG (γ-chain specific) antibodies. Inhibition was calculated as described under "Experimental Procedures." *Closed circles*, inhibition with ssDNA; *open circles*, inhibition with poly(IC). *C*, inhibition of the binding of murine and human autoantibodies to NF45·NF90 by ssDNA or poly(IC) was tested. *Open circles*, sera from pristane-treated mice with anti-NF90·NF45 antibodies; *closed circle*, human autoimmune serum YM. *D*, inhibition of the binding of individual sera to NF90 by ssDNA (*x* axis) and poly(IC) (*y* axis). *Open circles*, murine autoimmune sera; *closed circle*, patient YM serum.

(62% identical) (17, 24). RNA helicase A, a 140-kDa dsRNA-binding factor that interacts selectively with VA RNA_{II}, also appears to be the target of autoantibodies in pristane-induced lupus as well as human autoimmune disease.[3]

Another dsRNA-binding factor, p110, had an identical N-terminal amino acid sequence (Fig. 2) and exhibits immunological cross-reactivity with NF90 (Fig. 6). This protein could be either a precursor or an alternative form of NF90 or a different protein, possibly MPP4, which has a size of ~110 kDa (17). The relationship between NF90, MPP4, and p110 is uncertain because the full-length sequence of MPP4 has not been reported, and most antisera recognize both 90 and 110 kDa proteins. In view of the striking sequence homology between the revised NF90 cDNA sequence and the partial MPP4 sequences,[4] it is very possible that NF90 and p110 are transcribed from the same mRNA. The p110-specific serum shown in Fig. 5*A* (*lane 3*) may be useful for investigating this possibility.

The p130 and p80 dsRNA-binding autoantigens remain unidentified. There are several other proteins with sequence homology/similarity to the dsRNA-binding motifs of NF90, including dsRNA adenosine deaminase (2). This protein has 150- and 110-kDa isoforms, the former potentially a candidate for p130. However, the interferon-dependent expression of the 150-kDa dsRNA adenosine deaminase (2) argues against that possibility. There are numerous additional proteins exhibiting alignment scores <40, including ribonuclease III, protamine-1 RNA-binding protein, HIV-1 trans-activation-responsive RNA-binding protein, and PKR.

Although relatively common in certain strains of mice, autoantibodies to NF90·NF45, p110, p130, and p80 are unusual in humans. We screened over 1200 sera and found only two with anti-NF90·NF45 autoantibodies. One of the patients had scleroderma and the other systemic lupus erythematosus-rheumatoid arthritis overlap syndrome without features of scleroderma. Labrador *et al.* (25) recently have reported three additional patients with rheumatoid arthritis and features of scleroderma who produced autoantibodies against proteins of 105, 95, and 42 kDa (25). Exchange of sera suggests that these antigens are NF90·NF45 and p110.[5]

NF45 Forms Complexes with NF90 and p110—NF90 is thought to dimerize with NF45 (10, 11), an interpretation consistent with the co-immunoprecipitation data presented here (Fig. 5). Interpretation of the interactions of p110 with other dsRNA-binding factors is complicated by the immunological cross-reactivity of p110 with NF90 (Fig. 6). Nevertheless, by analyzing autoantibody production longitudinally, we found that autoantibodies against p110 occasionally developed in the absence of anti-NF90 and that they co-immunoprecipitated NF45 (Fig. 5*A*). Moreover, purified anti-NF45 antibodies co-immunoprecipitated p110. We conclude that like the homologous NF90, p110 may dimerize with NF45. Labrador *et al.* (25) also described complex formation (25). Based on immunoprecipitation with affinity purified antibodies, these investigators concluded that all three components form a single complex. Immunological cross-reactivity of anti-NF90 and p110 complicates the interpretation of this experiment, however. Although we cannot exclude a trimer, the present data (Fig. 5) are more compatible with the alternative explanation that there are both NF90·NF45 and p110·NF45 dimers.

At present, there is no evidence that p130 forms a stable complex with NF90, NF45, or p110. EB serum immunoprecipitated p110, NF90, and NF45 but not p130 (Fig. 1). Even under low salt conditions, co-immunoprecipitation was not seen (data not shown). Moreover, p130 was eluted from poly(IC)-agarose at lower salt than p110, NF90, or NF45. Thus, if an interaction exists, it is either transient or else unstable under the conditions used for immunoprecipitation.

Nucleic Acid-binding Properties of NF90·NF45, p110, and

[3] H. Yoshida, M. Satoh, and W. H. Reeves, manuscript in preparation.

[4] P. N. Kao, unpublished data.

[5] C Gelpi, personal communication.

p130—The NF90·NF45 complex has sequence-specific DNA binding (10, 11) as well as nonspecific and VA RNA_{II}-selective dsRNA binding activity (8). The present data indicating strong binding to poly(IC) and dsDNA from calf thymus (Fig. 7) are consistent with the previous reports. The p110 protein, probably in the form of a p110·NF45 dimer, showed similar nucleic acid binding properties but bound to ssDNA more efficiently than did NF90·NF45. In contrast, p130 bound efficiently to poly(IC), although less tightly than p110, NF90, or NF45, and also bound weakly to dsDNA but not ssDNA (Fig. 7). These data suggest that some dsRNA-binding autoantigens can interact with DNA as well as RNA. Other proteins with dual RNA and DNA binding activity have been described (26–28). Little is known about the mechanisms of dsRNA or DNA binding, although it is possible that the dsRNA-binding motifs of NF90 interact with dsRNA in a manner analogous to that of PKR (29). This may account for the high avidity of the NF90·NF45 interaction with dsRNA. Interestingly, the antibody inhibition studies (Fig. 8) suggest that the site(s) contacting dsRNA differ from those contacting DNA. The same is true of the DNA and RNA-binding sites of dsRNA adenosine deaminase (28). However, the domain-mediating interactions of NF90·NF45 with DNA have not yet been defined.

Relevance to Autoimmune Disease—The binding of dsDNA and dsRNA to p110, NF90, and NF45 may have implications for the pathogenesis of autoimmune disease. Because these proteins are constitutively expressed, they could play an early role in the cellular mechanisms for sensing ds nucleic acids resulting from viral infection or the escape of genomic or mitochondrial DNA into the cytoplasm in injured cells. Transfection of either dsRNA or dsDNA induces the expression of several genes involved in antigen processing and presentation, including class I and class II major histocompatibility complex antigens (30). Moreover, activation of the Jak/Stat, NF-κB, and MAPK systems is induced by transfection of dsRNA, potentially establishing a proinflammatory cytokine milieu conducive to the development of autoimmunity. There is considerable evidence that viral infections can promote autoimmunity (31–33). This may reflect the production of proinflammatory cytokines such as interleukin-12 (34), molecular mimicry (35), and/or the binding of virally encoded proteins to self-antigens (36). The possibility exists that binding of viral dsRNA to NF90·NF45, p110, or p130 might induce autoimmunity to these proteins, analogous to the development of high levels of autoantibodies to p53 in mice immunized with complexes of mouse p53 and SV40 large T antigen (36). The adjuvant-like properties of dsRNA also could promote autoimmunity.

In summary, a family of constitutively expressed dsRNA-binding proteins is recognized by autoantibodies produced in systemic autoimmune disease. Using the autoimmune sera, some of these proteins were shown to assemble into complexes that bind DNA as well as dsRNA. In some cases, DNA but not dsRNA inhibited autoantibody binding to NF90. In other cases, the reverse was true, suggesting that different sites mediate the interactions with DNA and dsRNA. Although the function of these proteins remains unclear, their constitutive expression and interactions with VA RNA_{II} and possibly other highly structured viral RNAs permit speculation that they may play a role in sensing the presence of viral infection.

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