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Recombinant 70-kD protein used for determination of autoantigenic epitopes recognized by anti-RNP sera

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SUMMARY

A panel of high- and low-titre anti-RNP-positive patient sera was tested for reactivity with human snRNP proteins. The U1 snRNP-specific 70-kD peptide was found to be the most prominent RNP antigen recognized by high-titre anti-RNP sera, mainly found in patients with mixed connective tissue disease (MCTD). The reactivity with the 70-kD protein was further analysed with recombinant fusion proteins containing different segments of the protein. One major and two minor antigenic regions were found. Most patient sera, both with high- and low-titre anti-RNP antibodies, only recognized the major region. The most aminoterminal region, showing partial sequence homology with a mouse retroviral p30 gag protein, contained an epitope that was recognized by one serum only.

Keywords autoantibodies cDNA expression vectors mixed connective tissue disease snRNP

INTRODUCTION

In 1972 mixed connective tissue disease (MCTD) was suggested as a distinct rheumatic syndrome sharing clinical features with systemic lupus erythematosus (SLE), progressive systemic sclerosis and polymyositis (Sharp *et al.*, 1972). In the initial study the most frequently reported symptoms in MCTD were polyarthralgia or arthritis, diffuse swelling of the hands (puffy hands) and two-phase Raynaud's phenomenon. In addition, the patients were characterized by the presence of high serum titres of an anti-nuclear antibody, called anti-RNP, exhibiting a speckled nuclear immunofluorescence pattern. Anti-RNP antibodies are also found in sera from patients with SLE, but usually in lower titres and in conjunction with another specificity, anti-Sm (Tan & Kunkel, 1966).

For detection of these antibodies, salt-extractable nuclear antigen (ENA) and immunological tests such as immunodiffusion (Tan & Kunkel, 1966), passive haemagglutination (Sharp *et al.*, 1976), and counter-immunoelectrophoresis (CIE) (Tan & Kurata, 1976) have been employed. By these methods, anti-RNP specificity has been defined with reference sera and distinguished from anti-Sm by differential RNAse sensitivity (Sharp *et al.*, 1972). More recently, using whole cell extracts for immunoblotting and immunoprecipitation, more precise information has been obtained on the specific peptides that the antibodies react with. Both types of antibodies are directed against intranuclear RNA–protein complexes involved in splicing, called U snRNPs (Lerner & Steitz, 1979). Several studies have shown that anti-RNP antibodies react with the U1 snRNP-specific peptides denoted 70-kD A and C (Pettersson *et al.*, 1984; van Venrooij & Habets, 1985). Of these, the 70-kD peptide seems to be the most prominent RNP antigen recognized by MCTD sera (Habets *et al.*, 1985; Pettersson *et al.*, 1986; Takeda *et al.*, 1989). It has been reported that anti-RNP antibodies could recognize a part of the 70-kD protein with sequence homologies to a mouse retroviral antigen, the p30 gag protein (Query & Keene, 1987). This cross-reaction was suggested as a clue to the potential involvement of a retrovirus in the induction of autoantibodies and possibly autoimmune disease. In an attempt to characterize more closely the autoantibody response against the 70-kD RNP antigen and to determine whether reactivity against the p30 gag homologous region is a general anti-RNP sera capability, we have examined sera from patients with low and high titres of anti-RNP antibodies using recombinant antigen.

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MATERIALS AND METHODS

Patients

Between 1980 and 1988, sera from patients admitted to the Department of Rheumatology, Huddinge University Hospital with symptoms or signs indicating inflammatory connective tissue disease were analysed for the presence of anti-nuclear antibodies (ANA). All sera exhibiting a speckled nuclear immunofluorescence pattern were routinely analysed by CIE for the presence of antibodies reacting with ribonuclease-sensitive RNP. Sera from patients with Raynaud's phenomenon, puffy hands, myositis and Sjögren's syndrome were also analysed for anti-RNP antibodies.

Table 1. Summary of clinical and serological data on the 28 anti-RNP-positive patients

Patient no.	Sex	Age (yr)	Diagnosis	RP	A	M	PP	SR	PH	PF	NS	R	OH	SD	PS	SI	LA	HD	Er	anti-RNP	Therapy
1	M	37	SLE	—	—	—	+	—	—	—	—	+	—	—	—	—	—	—	—	1/160	CS
2	M	49	DLE	—	—	—	na	+	na	na	—	na	na	na	na	—	—	—	na	1/40	—
3	F	22	MCTD	+	—	na	—	na	na	na	na	—	na	—	—	na	+	—	na	1/160	—
4	F	52	MCTD	+	—	—	—	+	+	—	—	—	+	—	+	—	—	—	—	> 1/640	CS
5	F	23	SLE	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1/2	ASA
6	F	64	MCTD	na	na	na	na	+	na	na	—	—	na	na	na	na	na	—	—	1/40	CS
7	F	22	UCTD	+	—	—	—	—	+	—	—	+	—	—	—	—	na	—	—	1/640	NSAID
8	M	60	MCTD	+	+	—	—	—	+	—	—	—	—	—	—	+	—	—	na	> 1/640	NSAID
9	F	52	MCTD	+	+	—	—	—	—	—	—	—	—	—	—	+	—	—	—	1/640	NSAID
10	F	28	MCTD	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	> 1/640	—
11	F	38	RP	+	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—	1/10	—
12	F	25	MCTD	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	> 1/640	—
13	M	23	UCTD	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	> 1/640	—
14	F	66	SLE	na	na	—	—	+	—	—	+	—	na	na	na	na	na	—	na	1/2	CS
15	F	46	RA	+	+	—	—	+	—	—	—	—	—	—	—	+	+	—	+	1/40	NSAID
16	F	38	MCTD	+	+	—	—	—	+	—	—	—	—	—	—	—	+	—	+	1/10	—
17	F	73	MCTD	+	—	—	—	—	+	+	—	—	—	—	—	+	—	—	—	1/320	CS
18	F	24	MCTD	—	—	—	—	+	+	—	—	—	—	—	—	—	—	+	na	1/320	—
19	F	27	RP	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	na	1/2	NSAID
20	F	55	SS	—	na	na	—	+	—	—	+	na	na	na	na	+	na	—	na	> 1/640	CS
21	F	32	MCTD	+	+	—	—	na	+	+	na	na	+	+	—	—	—	—	na	> 1/640	NSAID
22	F	27	SLE	—	—	—	—	+	+	—	—	—	—	—	—	—	—	—	na	1/2	—
23	F	23	MCTD	+	+	+	—	—	+	—	—	—	—	—	—	—	—	—	na	> 1/640	NSAID
24	F	44	SLE	+	—	na	—	+	+	—	na	na	na	—	—	—	—	—	na	1/40	NSAID
25	F	40	UCTD	+	—	—	—	—	—	—	—	—	—	—	—	+	—	—	na	1/160	—
26	F	57	MCTD	+	—	—	—	+	+	—	—	—	—	+	+	+	na	—	na	1/40	—
27	M	57	MCTD	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	na	> 1/640	—
28	F	65	SS	—	—	—	—	+	—	—	+	na	—	na	—	+	—	—	na	1/2	—

RP, Raynaud's phenomenon; A, arthritis; M, myositis; PP, pleuritis/pericarditis; SR, skin rush; PH, puffy hands; NS, nervous system involvement; R, renal disease; OH, oesophageal hypomotility; SD, sclerodactyly; PS, proximal scleroderma; SI, sicca symptoms; LA, lymphadenopathy; HD, haematologic disorder; Er, radiographic erosions; CS, corticosteroids; na, not assessed; ASA, acetyl salicylic acid; NSAID, non-steroidal anti-inflammatory drugs.

Sera from approximately 300 patients were examined by CIE on one or several occasions. Sera from 28 patients (23 women, five men, aged 22–73 years) gave a positive reaction for anti-RNP antibodies. Both low- and high-titre sera were represented. Sixteen sera had a high anti-RNP titre ($\geq 1/160$) and the remaining 12 had a low titre ($\leq 1/40$) using CIE with rabbit thymus and human spleen as antigens. Diagnoses and clinical data on the 28 patients whose sera contained anti-RNP antibodies are summarized in Table 1.

Diagnoses were based on the American Rheumatism Association criteria for SLE (Tan *et al.*, 1982) and rheumatoid arthritis (Ropes *et al.*, 1958). For a diagnosis of MCTD the criteria proposed by Alarcon-Segovia (1976) and Alarcon-Segovia & Cardiel (1989) were used; a diagnosis of Sjögren's syndrome was based on the criteria proposed by Manthorpe *et al.* (1986). The three patients not fulfilling any of the above proposed criteria were described separately under the heading unclassified connective tissue disease (UCTD). All three had Raynaud's phenomenon. In addition, one had puffy hands, one had synovitis of small joints and a third patient had dryness of the mouth and impaired salivation rate—thereby exhibiting one or more features characteristic of MCTD.

Detection of snRNP peptide antigens by immunoblotting

Nuclear extracts made from HeLa cells (Dignam, Lebovitz & Roeder, 1983) were separated on 10% SDS–PAGE, transferred electrophoretically to nitrocellulose (Towbin, Staehlin & Gordon, 1979) and probed with patient sera diluted 1/500. Bound antibodies were visualized with alkaline phosphatase-conjugated anti-human IgG secondary antibodies (Dako) diluted 1/2000 using the substrates nitroblue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma). The different snRNP peptides were identified using reference sera (Pettersson *et al.*, 1986) and monoclonal antibodies Y12 (Lerner *et al.*, 1981) and 2.73 (Billings *et al.*, 1982).

Isolation and characterization of a human 70-kD cDNA clone

The 70 kD RNP clones were obtained by screening a human placenta cDNA library inserted in the lambda phage expression vector λgt11 (Clontech) with anti-RNP sera from MCTD patients. Positive plaques were identified, rescreened and plaque-purified (Huynh, Young & Davis, 1985). The identification of the 70 kD clones was supported by staining with positive and negative control sera. The cDNA insert of the clone producing the largest fusion protein was excised with the restriction enzyme *Eco*RI (Pharmacia), ligated into the *Eco*RI site of the plasmid vector pGEM 3 (Promega) and mapped for restriction enzyme sites (Maniatis, Fritsch & Sambrook, 1982). The ends were sequenced by the dideoxy method (Sanger & Coulson, 1975) adapted for double-stranded plasmids (Gem-Seq, Promega). Restriction site mapping and sequence analysis demonstrated that the presently used clone corresponded to nucleotides 1398–2667 in the 70-kD protein cDNA sequence published by Theissen *et al.* (1986).

Construction of a series of expression clones

In order to obtain recombinant 70-kD protein, the cDNA insert was subcloned into the *Eco*RI site of the expression vector pEX2 (Stanley & Luzio, 1984), obtained from Boehringer-Mannheim. The recombinant proteins were expressed as 70K-cro-β-galactosidase fusion proteins. The initial construct containing the 1·3 kb insert was called pEX70K1. From it, deletion clones pEX70K2 to pEX70K11 were derived, mainly by using compatible restriction sites in the insert and the vector. The fragments of the initial cDNA clone contained in the different constructs are depicted in Fig. 1.

Expression of recombinant proteins

The pEX constructs were transformed into *Escherichia coli* N4830 cells (Gottesman, Adhya & Das, 1980). Expression of the recombinant fusion protein was induced by shifting the culture from 30°C to 42°C for 2 h (Stanley & Luzio, 1984). Cells from induced cultures were spun down and boiled for 5 min in 5% SDS sample buffer. The proteins were separated on 7·5% SDS–PAGE gels and stained with Coomassie blue (Laemmli, 1970). Fusion proteins were identified by a shift in the apparent molecular weight of the recombinant cro-β-galactosidase protein relative to the protein produced by the pEX vector alone.

Detection of recombinant 70 kD antigenic epitopes by immunoblotting

The proteins from induced cells were separated on 7·5% SDS–PAGE gels, transferred electrophoretically to nitrocellulose and probed with either an anti-β-galactosidase monoclonal (Promega) at a 1/20 000 dilution or human sera at a dilution range between 1/5000 and 1/10 000. The anti-β-galactosidase staining

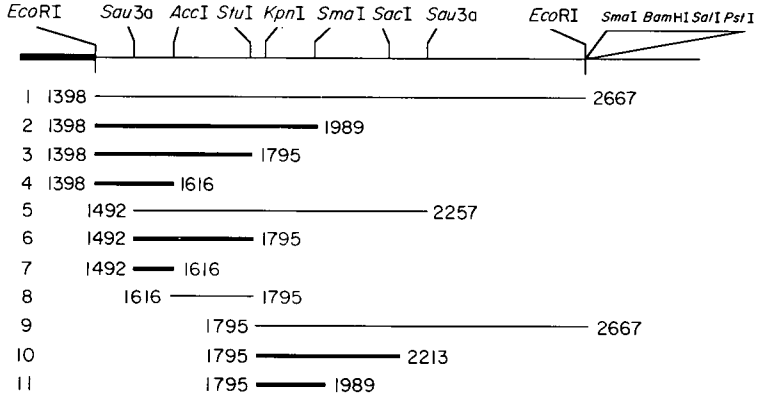

Fig. 1. Restriction map of cloned 70-kD cDNA fragments inserted into pEX2. The different constructs are denoted pEX70K 1–11. Clones used in this report are indicated by thick bars. The numbers at each end of the cDNA fragments refer to the position in the sequence published by Theissen *et al.* (1986).

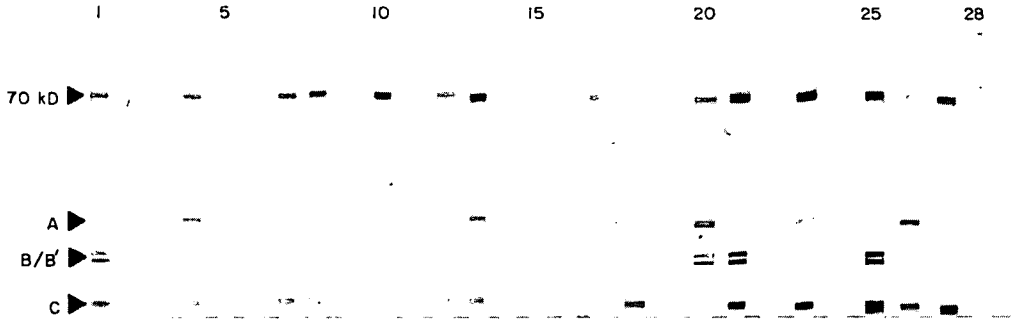

Fig. 2. Western blot with HeLa cell nuclear extract as antigen probed with the 28 different patient anti-RNP sera.

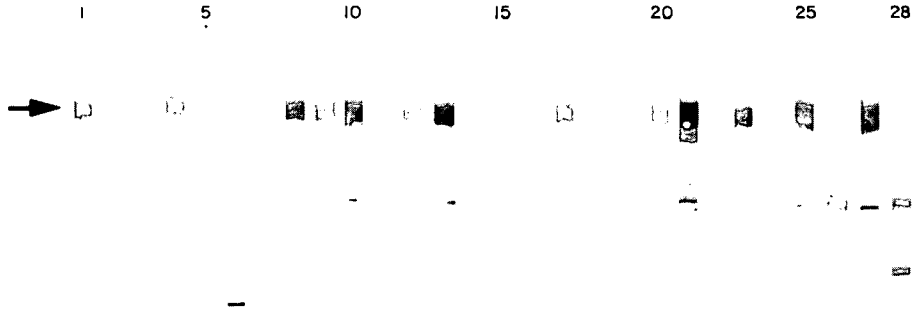

Fig. 3. Western blot with the recombinant 70-kD fusion protein pEX70K 2 as antigen. The blot is probed with the same set of sera as in Fig. 2. The arrow denotes the position of the fusion protein.

Table 2. Diagnoses of patients with low (≤1/40) and high (≥1/160) anti-RNP titres against ENA as measured with counter-immunoelectrophoresis

	Anti-RNP titre	
Diagnosis	≤1/40	≥1/160
Systemic lupus erythematosus	4	1
Rheumatoid arthritis	1	—
Sjögren's syndrome	1	1
Mixed connective tissue disease	3	11
Discoid lupus erythematosus	1	—
Mb Raynaud	2	—
Unclassified connective tissue disease	—	3
Total	12	16

was used to define the position of the recombinant fusion proteins on the blots.

RESULTS

Detection of snRNP peptide antigens with high- and low-titre anti-RNP sera by immunoblotting

The 28 anti-RNP sera used in this study were selected from a group of about 300 ANA-positive patient sera. Sixteen sera had a high anti-RNP titre, the remaining 12 had a low titre. Fifteen of the 16 high-titre anti-RNP sera were strongly reactive with the 70-kD protein, whereas five of the 12 low-titre sera reacted with the 70-kD protein on Western blots (Fig. 2). Seven of 12 low titre sera did not recognize the 70 kD protein. Reactivity against the A protein was found in six high-titre anti-RNP sera and in two low-titre sera. The B/B′ proteins were recognized by eight sera, including low- and high-titre sera. The C protein was recognized by 13 of 16 high-titre sera and by two of 12 low-titre anti-RNP sera.

Relationship between reactivity with individual snRNP proteins and diagnosis

Of the 16 high-titre sera, 11 were obtained from patients diagnosed as having MCTD (Table 2). Ten of these 11 MCTD sera displayed strong reactivity against the 70-kD protein. The MCTD serum not reacting with the 70-kD protein (patient serum no. 18; Fig. 2) reacted with the C protein.

Specificity of anti-70 kD antibodies assayed with recombinant 70-kD antigen

The 28 anti-RNP sera were used for blotting against recombinant antigens corresponding to different fragments of the 70-kD protein. The results are shown in Figs 3 and 4 and summarized in Table 3. Only one serum (patient serum no. 21) recognized the fusion protein produced by construct pEX70K4, which covers nucleotides 1398–1616. With this serum there was no reaction towards the pEX70K7 antigen encoded by nucleotides 1492–1616. Fourteen sera showed the same type of reactivity to the three recombinant antigens produced by pEX70K2 (Fig. 3), pEX70K3 and pEX70K6 sharing the cDNA nucleotides 1492–1795. None of these sera reacted with the fusion protein produced by pEX70K7. Three sera recognized the recombinant 70-kD proteins encoded by constructs pEX70K10 and

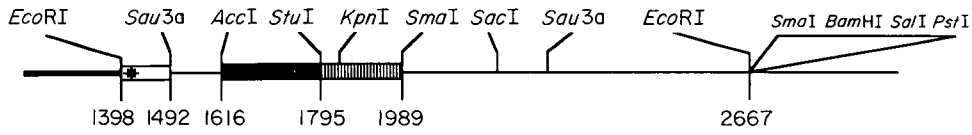

Fig. 4. Localization of cDNA regions, encoding epitopes on the recombinant 70-kD protein. Open bar, nucleotides 1398–1492: region encoding epitopes recognized by one serum; solid bar, nucleotides 1616–1795: region encoding epitopes recognized by 14 sera; hatched bar, nucleotides 1795–1989: region encoding epitopes recognized by three sera.

*The aminoterminal region with partial homology to a mouse p30 gag protein, nucleotides 1398–1454. The numbers refer to the position in the published 70-kD sequence (Theissen *et al.*, 1986).

Table 3. Western blot results using HeLa cell nuclear extract and six different recombinant fusion proteins encoded by deletion clones of the 70-kD cDNA

Serum no.	Nuclear extract (70 kD)	pEX70K constructs					
		2	3	4	6	10	11
1	+++	+++	+++	–	+++	+++	+++
2	–	–	+	–	–	–	–
3	++	+	++	–	–	–	–
4	+++	+++	+++	–	++	–	–
5	–	–	+	–	–	–	–
6	–	–	+	–	–	–	–
7	+++	+++	++++	–	++	–	–
8	++++	++++	++++	–	++	–	–
9	++	+++	++++	–	+	–	–
10	++++	++++	++++	–	++++	–	–
11	+	+	+	–	–	–	–
12	+++	+++	++++	–	++	–	–
13	++++	++++	++++	–	++++	–	–
14	–	–	0	–	–	–	–
15	+	+	0	–	–	–	–
16	+	+	0	–	–	–	–
17	+++	+++	+++	–	++	–	–
18	–	–	–	–	–	–	–
19	–	–	0	–	–	–	–
20	+++	+++	+++	–	++++	–	–
21	++++	++++	++++	++++	+++	–	–
22	+	–	0	–	–	–	–
23	++++	++++	++++	–	++++	++++	++++
24	–	–	0	–	–	–	–
25	++++	++++	++++	–	++++	–	–
26	++	+	–	–	–	–	–
27	++++	++++	+++	–	++	++	++
28	–	–	0	–	–	–	–

The relative intensity of the signals on the Western blots were scored in the range of – (negative) to ++++. Seven seras were not probed with recombinant construct pEX70K3 (0).

pEX70K11, which have nucleotides 1795–1989 in common. These three sera also reacted with the pEX70K2 fusion protein.

Relationship between reactivity with different recombinant 70-kD fragments and anti-RNP titre

Of the 16 high-titre anti-RNP sera, 15 reacted with the 70-kD fragments encoded by the larger clones sharing nucleotides 1616–1795. However, only 14 of these sera recognized pEX70K6. Four of these sera (sera 1, 21, 23 and 27) also contained antibodies reacting with other parts of the 70-kD protein.

Seven of the 12 low-titre sera exhibited a weak reactivity with one or both of the recombinant 70-kD constructs pEX70K2 and pEX70K3, whereas the remaining five low-titre sera were negative. No low-titre sera reacted with the recombinant construct pEX70K6.

DISCUSSION

High titre of RNAse-sensitive anti-RNP antibodies is a serological characteristic of MCTD. It is also found in some patients with UCTD, especially in cases with one or more clinical manifestations characteristically present in MCTD, notably two-phase Raynaud's phenomenon or puffy hands. When found in well-defined connective tissue diseases such as SLE or

Sjögren's syndrome, the titre is usually lower and the antibodies often appear together with other autoantibody specificites.

Since high and low titre antibody categories seem to select patient populations with different clinical manifestations, the question arises whether anti-RNP specificity differs between high- and low-titre sera. If so, this might indicate a different immunization with respect to anti-RNP autoantibodies.

A limitation in the analysis of anti-RNP antibody specificity, using immunoblotting with nuclear extract as antigen, is the inability to resolve reactions against different parts of the 70-kD protein. To circumvent this problem, a human 1270-bp long cDNA clone, encoding a major part of the 70-kD protein, was isolated. This clone corresponded to nucleotides 1398–2667 in the previously reported 70-kD protein cDNA sequence (Theissen *et al.*, 1986).

Of the 450 amino acids making up the 70-kD protein, our clone encoded 374 (83%) of the total protein. Therefore, the possibility that antigenic regions existed in the part of the protein not encoded by the cDNA could not be excluded. However, other investigators (Guldner *et al.*, 1988) have not found evidence for antigenic regions in this amino terminal part of the protein.

The recombinant fusion protein produced in *E. coli* was recognized by all high-titre anti-RNP sera tested except one (no. 18). When comparing immunoblots probed with the 28 patient sera, using nuclear extract or recombinant fusion protein as antigen, the results were congruent. One group of sera reacted strongly with 70 kD in HeLa nuclear extracts and with recombinant fusion proteins and another gave a weaker reaction with both types of antigen. Obviously, a substantial fraction of all patients anti-RNP antibodies recognizing eukaryotic 70 kD also reacted with the recombinant protein. The only high-titre RNP serum not reacting with recombinant fusion protein was demonstrated to react only with the C-peptide on the nuclear extract immunoblot. Interestingly, this patient was the only one in the MCTD group without Raynaud's phenomenon.

The immunoblotting procedure requires both boiling and the presence of a strong detergent, SDS. It may be that the recombinant antigen treated in this fashion is not recognized by autoantibodies directed against conformational epitopes on the 70-kD protein. However, the fusion proteins have also been purified without SDS, and used as antigen in an ELISA. Results obtained from this type of assay are also in accordance with the immunoblotting results (Takeda *et al.*, personal communication).

The blotting assay using recombinant 70-kD antigen seems to be more sensitive than blots based on authentic 70-kD protein. The nuclear extract immunoblots were performed with the sera diluted 1/500, whereas the recombinant antigens were tested with sera diluted at least 1/5000 and it still gave a stronger reaction. Thus, very weak sera might be scored as negative against 70 kD itself but give a weakly positive reaction when tested against recombinant antigen. An example of this is shown in Table 3. Three sera (nos. 2, 5 and 6) demonstrated no 70 kD reaction in nuclear extract immunoblots but gave weakly positive reactions against one of the recombinant proteins encoded by pEX70K3. However, these sera did not react with the antigens encoded by any of the other pEX70K clones, including the overlapping and slightly longer pEX70K2 clone. The piece of DNA added on to pEX70K2 in comparison with the shorter pEX70K3 encodes a region of the 70-kD protein which is expressed as the carboxyterminal part of the recombinant antigen. It is largely composed of charged amino acid residues like arg, asp and glu. These could interfere with antibody binding through their charge or block access to an antigenic site by affecting conformation and thus make a weakly positive reaction undetectable.

Three different antigenic regions were found on the recombinant 70-kD protein (Fig. 4). One antigenic region, encoded by pEX70K4, was recognized only by one patient serum which also reacted with another 70-kD antigen. This region was proposed (Query & Keene, 1987) to be the RNP epitope, containing a sequence homology with a mouse retroviral gag protein.

A similar approach to the mapping of the 70 kD antigenic epitopes has been described (Guldner *et al.*, 1988), using an independently derived 70-kD clone and another expression vector system. These authors also demonstrated three regions of antigenicity. In their collection of 17 sera, only one serum showed reactivity against recombinant proteins encoded by sequences partly corresponding to pEX70K4. The infrequent recognition of the amino terminal part of the fusion protein, showing retroviral sequence homology, makes it unlikely that this region is the major RNP antigen.

The most frequently recognized region, which was recognized by all sera with high titres of anti-RNP antibodies except one, was localized on the central portion of the protein encoded by nucleotides 1616–1795. This part of the protein is hydrophobic, not especially charged and contains several aromatic amino acids. The region has been suggested as a possible RNA binding motif (Theissen *et al.*, 1986; Spritz *et al.*, 1987).

A less frequently recognized epitope is encoded by the sequence between nucleotides 1795–1989. Three sera out of 28 in the patient sera collection contained reactivity against this region. These patients had high-titre anti-RNP sera, one was diagnosed as having SLE and the others as being MCTD patients. This part of the protein has a rather extreme amino acid composition containing almost exclusively charged amino acids.

Two monoclonal anti-RNP antibodies of human (Chen *et al.*, 1988) and mouse (Billings *et al.*, 1982) origin also reacted with epitopes in this part of the protein when they were used in immunoblots against recombinant protein produced by the expression vector pEX70K10 (data not shown). When the 70-kD positive high- and low-titre sera were compared using pEX70K2 and pEX70K3, no difference with respect to the most frequently recognized 70-kD region was found at this level of resolution. However, pEX70K6 was recognized by 14 of 15 high-titre and no low-titre sera. Further studies are necessary in order to clarify whether this pattern of reactivity is a persistent finding or if the antibody reactivity changes during the clinical course of the diseases.

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