Insights Into Native Epitopes of Proliferating Cell Nuclear Antigen Using Recombinant DNA Protein Products

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Summary

A cDNA clone encoding full-length human proliferating cell nuclear antigen (PCNA) was used to generate a panel of in vitro translated labeled protein products with COOH-terminal deletions and to construct a set of fusion proteins with COOH- and NH_2-terminal deletions. A rabbit antiserum raised against an NH_2-terminal peptide, a well-characterized murine monoclonal antibody (mAb), and 14 human lupus sera with autoantibody to PCNA were analyzed for their reactivity with the constructs using both immunoprecipitation and immunoblotting techniques. The rabbit antiserum reacted in immunoprecipitation and immunoblotting with constructs containing the appropriate NH_2-terminal sequence and mAb reacted with a sequence from the midregion of PCNA. These experimentally induced antibodies also reacted with 15-mer synthetic peptides in enzyme-linked immunosorbent assay (ELISA). In contrast, none of the lupus sera reacted with synthetic peptides in ELISA. 9 of the 14 lupus sera also failed to react in Western immunoblotting with any recombinant fusion protein, although they all immunoprecipitated in vitro translated full-length protein. Four of the nine had variable patterns of immunoprecipitation with shorter constructs. The remaining five lupus sera were able to immunoprecipitate translation products as well as Western blot recombinant fusion proteins. From analysis of the patterns of reactivity of human lupus sera, it was deduced that the apparent heterogeneity of human autoantibodies to PCNA could be explained by immune response to highly conformational epitopes. These observations demonstrate that there might be special features in "native" epitopes of intranuclear antigens that are recognized by autoantibodies, and that these special features of native epitopes might not be present in prepared antigen used for experimental immunization. These features may be related to protein folding or to association of the antigen with other intranuclear proteins or nucleic acids, as might occur with antigens that are components of subcellular particles.

Proliferating cell nuclear antigen (PCNA)[1] is the target antigen for autoantibodies in the sera of ~3% of patients with SLE (1, 2). Although the autoantibodies occur in low frequency in lupus, they have become valuable reagents for the study of a key cellular component of the cell nucleus in the late G_1 and early S phase of the cell cycle (3–6). PCNA has been identified as an auxiliary protein of DNA polymerase δ (7–9), a multisubunit nuclear particle that is required for the replication of human (10, 11) and simian virus 40 DNA (10, 12) and frog chromosomal DNA (13).

The nature of the autoimmune response to PCNA has been of interest and has been facilitated by extensive information available concerning its molecular nature (7–9), identification of its function (10–14), and cloning of DNA that encodes the protein (15–17). Ogata et al. (18) analyzed the antigenicity of PCNA fragments generated by limited protease digestion of purified rabbit PCNA. Reactivity of a rabbit polyclonal serum raised against an NH_2-terminal peptide (rabbit antipeptide antibody [RAPAb]), two murine mAbs (19) raised against purified rabbit PCNA, and eight lupus sera with anti-PCNA activity were analyzed. From this study, antigenic domains reacting with lupus sera were shown to be located in both the NH_2- and COOH-terminal halves of PCNA, whereas two murine mAbs (19A2 and 19F4) reacted with a different domain located between the two lupus domains. Further differences between monoclonal and lupus antibodies have been demonstrated in functional assays, where autoan-

[1] *Abbreviations used in this paper:* FP, fusion protein; IPTG, isopropyl-B-thio-galactopyranoside; IVP, in vitro protein products; PCNA, proliferating cell nuclear antigen; RAPab, rabbit antipeptide antibody.

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tibodies were capable of inhibiting polymerase δ–associated DNA replication, but experimentally induced mAbs were ineffective (13, 14).

In an attempt to further define the nature of the autoimmune response, we have taken a molecular approach. A cDNA encoding full-length human PCNA was cloned and used to synthesize a panel of radiolabeled in vitro translation products and a series of recombinant fusion proteins (FPs) representing defined portions of the PCNA polypeptide. Using immunoprecipitation and immunoblotting techniques, reactivities of RAPAb, mAb 19A2, and 14 lupus sera were analyzed with the aim of gaining more insight into the nature of antigenic determinants recognized by human autoantibodies.

Materials and Methods

Source of Antibodies. RAPAb was raised against a synthetic peptide comprising amino acid residues 11–23 (18). The murine mAb 19A2 (IgM) was raised by immunization of BALB/c mice with purified rabbit PCNA in CFA and hybridomas derived from spleen cells (19). Both these experimentally induced antibodies to PCNA have been used previously and their cognate epitopes assigned to different *Staphylococcus aureus* V8 proteolytic fragments (18). In this manner, the 19A2 mAb had been shown to recognize a proteolytic fragment located in the midregion of PCNA separate from lupus autoantibody epitopes. Lupus sera used in this study were from our laboratory serum bank and from patients seen by Dr. Y. Takasaki (Juntendo University School of Medicine, Tokyo, Japan). These sera were defined as containing antibodies to PCNA by immunodiffusion analysis showing the presence of a precipitating antibody with complete immunological identity with standard reference serum, and by immunofluorescence showing nuclear staining that varied with different phases of the cell cycle, but that was most prominent in the G_1/S phase, as reported previously (1, 5, 6, 20, 21).

Synthetic Peptides. 26 overlapping 15-mer peptides covering the entire amino acid sequence of PCNA were synthesized by the method of simultaneous multiple peptide synthesis, as described previously (22). All peptides were synthesized as COOH-terminal amides and analyzed by reverse phase HPLC for purity, as reported in a recent publication (23). The amino acid sequence of the individual peptides with overlapping residues are shown in Table 1. The peptides were used for ELISA, according to a method described previously (21). Briefly, 96-well Immulon II plates (Dynatech Laboratories, Alexandria, VA) were coated with the peptides at a concentration of 0.4 μg per well overnight and incubated in a buffer containing 10% gelatin in order to block nonspecific binding sites. Lupus sera were tested at a dilution of 1:200 in serum diluent containing phosphate buffer (pH 7.4), gelatin (10%), BSA (1 mg/ml), and Tween-20 (0.05%). Peroxidase-labeled rabbit $F(ab)_2$ anti-human IgG antibody (Dakopatts A/S, Copenhagen, Denmark) was used diluted 1:200. For the mAb, a peroxidase-conjugated goat anti-mouse IgG/IgM (Caltag Laboratories, San Francisco, CA) was used diluted 1:15,000, and for the rabbit antiserum, a goat anti-rabbit peroxidase conjugate (Caltag Laboratories) was used diluted 1:200. The mAb was diluted in serum diluent described above and tested at concentrations from 0.1 to 25 μg/ml. The rabbit antipeptide serum was tested at dilutions from $1:10^2$ to $1:10^6$.

Cloning of PCNA. A cDNA encoding rat PCNA (15) was used to screen a λ gt11 cDNA library constructed from MOLT-4 human lymphoblastoma mRNA. One phage, 1–4.3, containing a 1.2-kb cDNA insert, was selected for further analysis. The insert was excised using partial EcoR1 digestion and subcloned into the plasmid Bluescript (Stratagene, La Jolla, CA). Three subclones were isolated that were used in this work. Plasmids pHPCNA15 and pHP5.7 contained the entire 1–4.3 cDNA insert extending from 114 bp 5′ of the initiator ATG to a poly(A) tail; the two plasmids contained the insert in opposite orientations within the vector. Plasmid pHPCNA10 contained a portion of the 1–4.3 cDNA insert extending from the 5′ untranslated region to the internal EcoR1 site at codon 127. The identity of the cDNA as human PCNA was verified by DNA sequencing of the termini of the clone and by restriction mapping and comparison with the sequence of Almendral et al. (16).

In Vitro Transcription and Translation. Plasmids were linearized by restriction digestion and used directly for in vitro transcription using T7 RNA polymerase (Promega Biotec, Madison, WI) according to the manufacturer's instructions. Plasmid pHPCNA15 was linearized with ClaI, Asp718, and BglII for synthesis of full-length, 211- and 150–amino acid derivatives of PCNA, respectively, while pHPCNA10 was linearized with ClaI, EcoRV, and NcoI to produce 128–, 85–, and 67–amino acid PCNA derivatives (see Fig. 2). Transcripts were quantitated by absorption at 260 nm and were used without further purification for in vitro translation. Radiolabeled in vitro protein products (IVPs) were synthesized by translation of T7 RNAs in a nuclease-treated rabbit reticulocyte lysate (Promega Biotec, WI) according to the manufacturer's instructions using ^{35}S-methionine (NEN Research Products, Wilmington, DE). Protein synthesis was monitored by SDS-PAGE and fluorography.

Fusion Proteins. A series of eight plasmids expressing β-gal/PCNA FPs were constructed in Bluescript. Plasmid pHP5.7, containing the PCNA cDNA in the same orientation as the β-galactosidase gene of Bluescript, was used to construct the expression derivatives. RE sites used for 5′ deletions were BssHII, BspMI, BalI, and StyI. RE sites for 3′ deletions were BglII, EcoRI, and BalI. Klenow fragment was used as needed to re-establish frame (24). DH1 bacteria were transformed with the plasmids, and FP production was induced by growing 5-ml bacterial cultures to an OD_{550} of 0.5. Isopropyl-B-thio-galactopyranoside (IPTG) was added to a 10-mM final concentration, and the culture was incubated overnight at 37°C. In the morning, the cultures were placed on ice for 5 min, the bacteria pelleted, resuspended in 200 μl of 1× Laemmli sample buffer, boiled for 5 min, run repeatedly through a 25-gauge needle until nonviscous, and then stored at −20°C.

Immunoprecipitation. Labeled synthetic IVPs were immunoprecipitated using staphylococcal protein A–coated sepharose beads (Pharmacia Fine Chemicals, Piscataway, NJ) adsorbed with antibody as previously described (20). Immunoprecipitated products were run on 15% SDS-PAGE gels, and labeled protein bands were visualized by fluorography.

Immunoblotting. Bacterial FP lysates from above (15 μl) were run on 15% SDS-PAGE, transferred to a nitrocellulose filter, and reactivity of RAPAb, 19A2, and SLE sera with the FPs was tested as previously described (18). Sera and antibodies were used at a 1:200 dilution and were preabsorbed overnight at 4°C with nontransformed bacterial lysate in a 100:1 ratio. Bacterial lysate was prepared from DH1 *Escherichia coli* containing Bluescript plasmid induced overnight with IPTG as above. A 100-ml culture was used to make 5 ml of lysate.

Results

Reactivity with Synthetic Peptides in ELISA. mAb 19A2 reacted only with peptide 12, which covers amino acid residues 111–125, and not with any of the other 25 synthetic peptides (Fig. 1). The reaction of 19A2 was inhibited 40–60% by pep-

Table 1. *PCNA 15-mer Synthetic Peptides*

Peptide No.	Amino acids	Amino acid sequence
1	1–15	MFEARLVQGSILKKV
2	11–25	ILKKVLEALKDLINE
3	21–35	DLINEACWDISSSGV
4	31–45	SSSGVNLQSMDSSHV
5	41–55	DSSHVSLVQLTLRSE
6	51–65	TLRSEGFDTYRCDRN
7	61–75	RCDRNLAMGVNLTSM
8	71–85	NLTSMSKILKCAGNE
9	81–95	CAGNEDIITLRAEDN
10	91–105	RAEDNADTLALVFEA
11	101–115	LVFEAPNQEKVSDYE
12	111–125	VSDYEMKLMDLDVEQ
13	121–135	LDVEQLGIPEQEYSC
14	131–145	QEYSCVVKMPSGEFA
15	142–155	SGEFARICRDLSHIG
16	151–165	LSHIGDAVVISCAKD
17	161–175	SCAKDGVKFSASGEL
18	171–185	ASGELGNGNIKLSQT
19	181–195	KLSQTSNVDKEEEAV
20	191–205	EEEAVTIEMNEPVQL
21	201–215	EPVQLTFALRYLNFF
22	211–225	YLNFFTKATPLSSTV
23	221–235	LSSTVTLSMSADVPL
24	231–245	ADVPLVVEYKIADMG
25	241–255	IADMGHLKYYLAPKI
26	247–261	LKYYLAPKIEDEEGS

Underlined residues represent sequences overlapping with preceding or succeeding peptide.

tide 12 at a concentration of 1 μg/ml, and 95% inhibition was obtained at 10 μg/ml. Other synthetic peptides were not inhibitory. Biochemically purified rabbit PCNA at concentrations of 0.2–2.0 μg/ml showed increasing degrees of inhibitions ranging from 25 to 95%, respectively. Rabbit antipeptide antibody reacted only with peptide 2 (Fig. 1), which comprises amino acid residues 11–25 (immunizing peptide was residues 11–23). Therefore, RAPAb reacted with the expected synthetic peptide, and 19A2 confirmed earlier studies that the epitope was located in the mid-region of PCNA (18, 21). In striking contrast to these results observed with experimentally induced antibodies, none of the 14 human lupus sera recognized any of the 26 synthetic peptides. With some of the peptides, there was a low noise background OD reading, but the same background reading was observed with eight normal human sera tested as controls. The absence of specific reactivity with synthetic peptides was not due to lack of binding of peptides to ELISA plates, since presence of each peptide on the plates was verified by direct colorimetric assay.

PCNA Clone. The restriction enzyme map for the 1.2-kb cDNA insert in plasmid pHPCNA15 is shown in Fig. 2. It is in agreement with the restriction enzyme map of the published sequence (16), except for a second BglII site located ~200 nucleotides distal to the stop codon in the 3′ untranslated region. Sequencing of the 5′ end of pHPCNA15 showed it to begin at nucleotide 15 of the sequence reported by Almendral et al. (16). This sequence was identical except for the substitution of a cytosine for a thymidine at position 61 in the 5′ untranslated region.

In Vitro Translation Products. Plasmids pHPCNA15 and

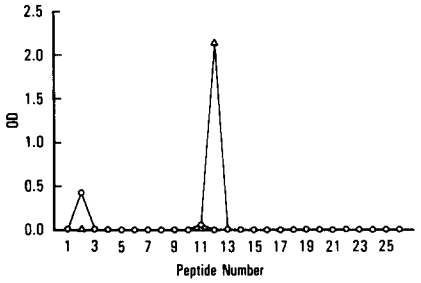

Figure 1. Reactivity in ELISA with 15-mer synthetic peptides. RAPAb (O) reacted with peptide 2, which contained the sequence of the peptide used for immunization. No other peptides reacted with RAPAb. The mouse monoclonal 19A2 (Δ) reacted only with peptide 12, a region of the PCNA protein that was inferred in previous studies to contain the reactive site.

pHPCNA10 were linearized by restriction enzyme digestion to produce templates for runoff in vitro transcription and translation, as described in Materials and Methods. Six protein products were synthesized, ranging from the full-length 261-amino acid "native" PCNA to a 67-residue NH_2-terminal fragment (IVPs 1–6) (Fig. 2). SDS-PAGE and fluorography of the IVPs show protein products of approximately 36, 34, 20, 14, 10 and 8 kD in Fig. 3, lanes *1–6*, (the 10- and 8-kD products ran with the dye front). Additional lower molecular weight polypeptides, designated IVP-F, were observed in conjunction with the two largest translation products (IVPs 1 and 2; Fig. 3, lanes *1* and *2*). As these bands were not present in controls and because of their lack of reactivity with RAPAb and positive reactivity with some lupus sera (see below), we interpreted them to be COOH-terminal fragments of PCNA.

FP Constructs. Eight FP cDNA constructs containing different portions of PCNA were prepared and expressed in

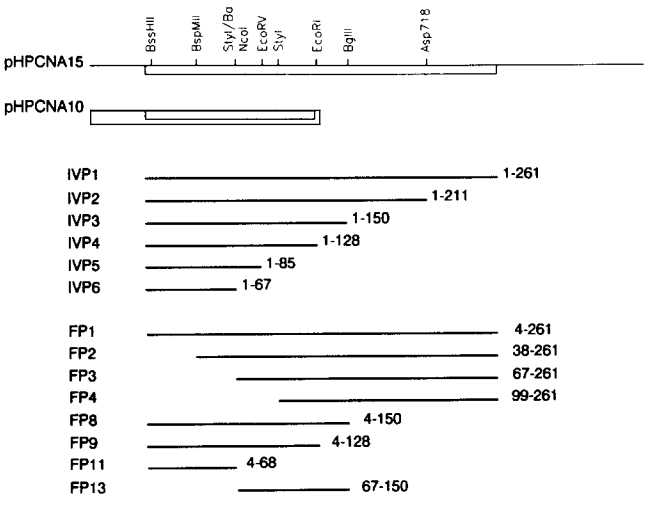

Figure 2. cDNA restriction enzyme map of PCNA and the recombinant protein products derived by in vitro transcription and translation (IVP1-IVP6), and expression FPs used to map fragments reactive with antibodies.

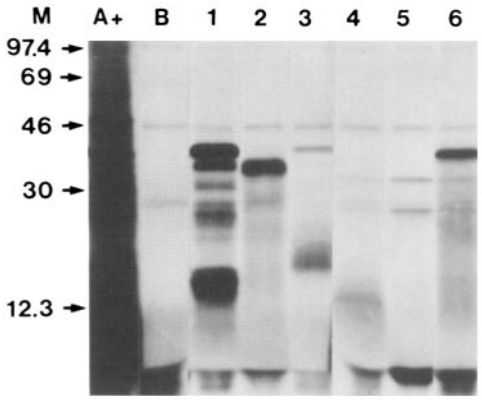

Figure 3. Fluorography in 15% SDS-PAGE of rabbit reticulocyte transcription/translation products from synthetic RNAs corresponding to constructs IVPs 1–6 in Fig. 2. M, molecular weight markers; A^+, Poly(A)$^+$ mRNA translation; B, without added mRNA; 1–6, IVPs 1–6 translation products. Some full-size PCNA is seen in lanes *3* and *6* due to incomplete digestion of full-length plasmid. Lower molecular weight products prominent in lane *1* are presumed to be degradation products of full-length protein.

E. coli (diagrammed in Fig. 2). FPs 1, 8, 9, and 11 were fused at codon 4 of the human PCNA sequence with a 32-codon fragment of β galactosidase. FPs 8, 9, and 11 had progressive 3′-terminal deletions in the PCNA sequence and terminated

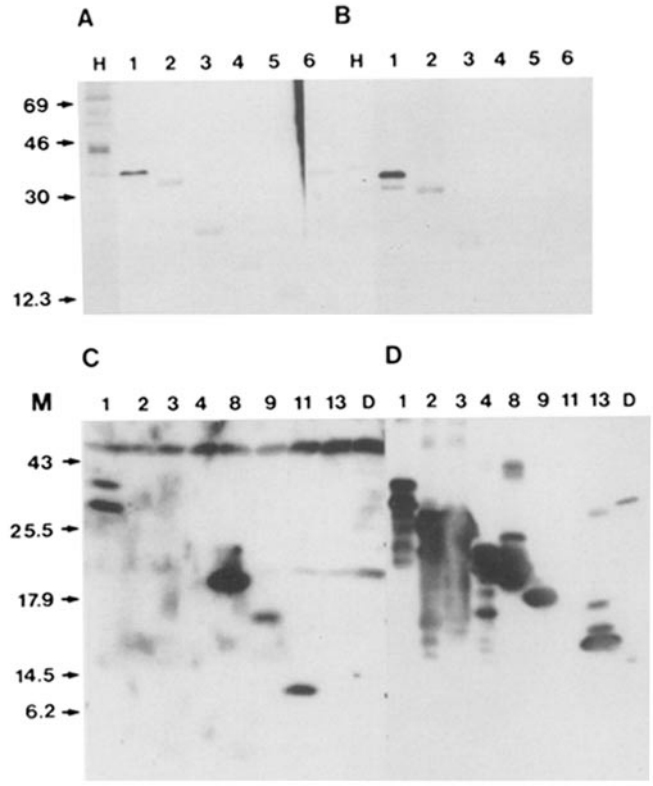

Figure 4. Immunoprecipitation of IVPs with (*A*) RAPAb; (*B*) mAb 19A2. Lane *H* is labeled HeLa whole cell extract, and lanes *1–6* are translation products 1–6, respectively. (*C* and *D*) Western blotting of fusion proteins with (*C*) RAPAb and (*D*) 19A2. Numbered lanes correspond to the fusion proteins in Fig. 2; (*D*) DH1 *E. coli* lysate; (*M*) molecular weight markers.

Table 2. *Immunoreactivity of IVPs and FPs of PCNA*

Protein product	Amino acid sequence	Reactivity with antibodies															
				SLE I					SLE II				SLE III			SLE IVA	SLE IVB
		RAPab	mAb 19A2	AI	YK	MI	KU	JO	CR	OK	CA	EB	PT	AK	YO	NE	FL
IVP 1	1–261	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
IVP 2	1–211	+	+	−	−	−	−	−	+	−	−	−	−	−	−	+	−
IVP 3	1–150	+	+	−	−	−	−	−	+	−	−	−	−	−	−	+	−
IVP 4	1–128	+	−	−	−	−	−	−	+	+	+	+	−	−	−	−	+
IVP 5	1–85	+	−	−	−	−	−	−	−	+	+	+	−	−	−	−	+
IVP 6	1–67	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
IVP-F*	Unknown	−	−‡	−	−	−	−	−	−	−	−	−	+	+	+	+	+
FP 1	4–261	+	+	−	−	−	−	−	−	−	−	−	+	+	+	+	+
FP 2	38–261	−	+	−	−	−	−	−	−	−	−	−	+	+	+	+	+
FP 3	67–261	−	+	−	−	−	−	−	−	−	−	−	+	+	+	+	+
FP 4	99–261	−	+	−	−	−	−	−	−	−	−	−	+	+	+	+	+
FP 8	4–150	+	+	−	−	−	−	−	−	−	−	−	+	+	+	+	+
FP 9	4–128	+	+	−	−	−	−	−	−	−	−	−	−	+	+	+	+
FP 11	4–68	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
FP 13	67–150	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	+

Results shown are immunoprecipitation for IVP and immunoblotting for FP.
* Lower molecular weight IVPs generated from full-length clone, possibly degradation products.
‡ Reactive only with the largest fragment of IVP-F.

with 6, 7, and 6 vector-derived codons, respectively. FPs 2, 3, 4, and 13 encoded proteins with progressive NH_2-terminal deletions in PCNA fused with a 30-residue NH_2-terminal leader derived from β galactosidase (Fig. 2). FP 13 encoded both NH_2- and COOH-terminal deletions. DNA sequencing across the deletion sites of FPs 1 and 4 confirmed the predicted nucleotide sequences.

Immunoprecipitation and Immunoblotting of RAPAb and 19A2. RAPAb, which was raised against synthetic peptide 11–23 of PCNA, immunoprecipitated all six IVPs, as expected (Fig. 4 *A*). Note that the lower molecular weight proteins detectable in Fig. 3, lane *1* (IVP-F) were not immunoprecipitated by RAPAb, suggesting that these lower molecular weight proteins lack this NH_2-terminal sequence. mAb 19A2 immunoprecipitated IVPs 1, 2, and 3, as expected, but not the shorter protein constructs (Fig. 4 *B*). Surprisingly, IVP 4, which consists of residues 1–128 and therefore contained the region 111–125, was not immunoprecipitated by 19A2 (Fig. 4 *B*). In addition to precipitation of full-length IVP 1, 19A2 precipitated a product that migrated just below the full-length construct (Figure 4 *B*, lane *1*).

In Western blotting, RAPAb was reactive with FPs 1, 8, 9, and 11 (Fig. 4 *C*), all of which contained amino acids 11–23, the peptide that was used in immunization. mAb 19A2 was reactive with FPs 1–4, 8, 9, and 13, but not with FP 11 (Fig. 4 *D*). These findings are also consistent with the observations showing that 19A2 reacted in a highly specific manner with peptide 111–125. It can also be observed from Fig. 4 *D* that 19A2 was reactive with many lower molecular weight bands of several FPs, suggesting that these products contained epitopic determinants present in peptide 111–125.

Reactivity of Lupus Sera. Specific patterns of reactivity of lupus sera with in vitro translated products and recombinant FPs allowed the autoantisera to be classified into four groups (Table 2). The first group of five sera immunoprecipitated the full-length native PCNA construct only and were not reactive with any of the shorter in vitro translated products (Fig. 5 *A*). Another feature of group 1 sera was that they were totally nonreactive in Western blots with any of the fusion proteins. The second group of four sera (SLE II) all immunoprecipitated full-length construct (IVP 1) but showed variable reactivity with the protein constructs containing COOH-terminal deletions (IVPs 2–5; Fig. 5 *B*). They were also nonreactive with the lower molecular weight fragments of IVP 1 (IVP-F). The immunoprecipitation pattern of serum O.K. in this group is depicted in Fig. 5 *B*, showing immunoprecipitation of full-length PCNA in lanes *1* and *3* (the latter due to residual undigested full-length cDNA) and IVPs 4 and 5 in lanes *4* and *5*, respectively. There was some variability in this group since serum C.R. was reactive with all

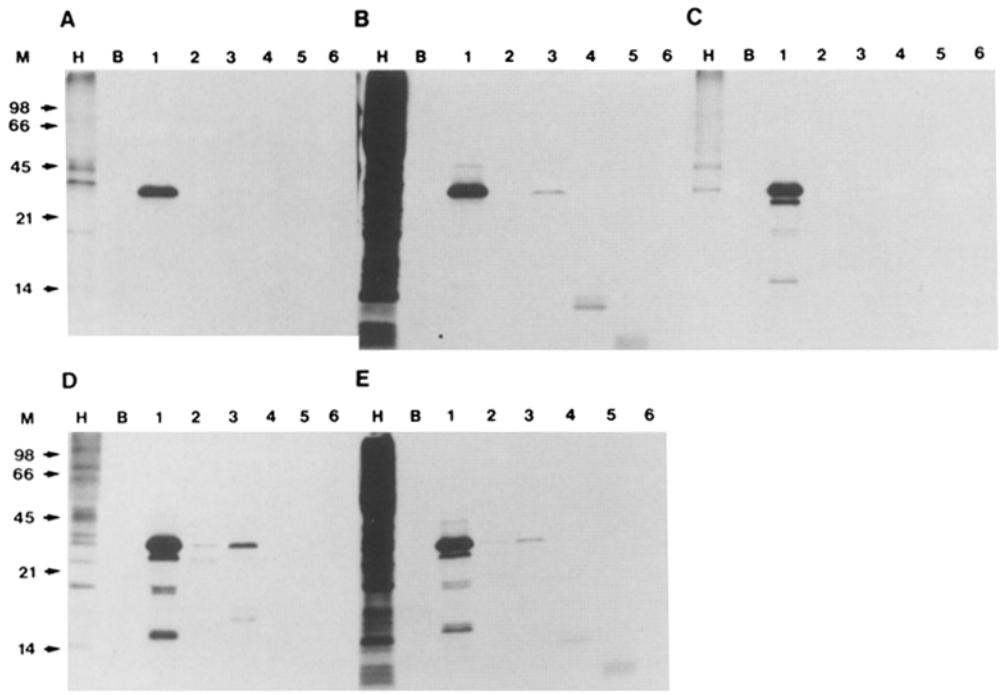

Figure 5. Immunoprecipitation of IVPs with lupus sera. (*A*) Representative group I serum AI; (*B*) representative group II serum OK; (*C*) representative group III serum AK; (*D*) representative group IVA serum NE; and (*E*) representative group IVB serum FL. Lane *H* is labeled HeLa whole cell extract, lane *B* is blank, where no serum was used in immunoprecipitation of IVP1. Lanes *1* and *6* are translation products IVPs 1–6, respectively. Immunoprecipitation of spontaneous degradation fragments of full-length protein (lane *1*) is observed in *C*, *D*, and *E*.

of the four longer constructs, while the three remaining sera, O.K., C.A., and E.B., were not reactive with the two intermediate constructs IVP 2 and 3, but were reactive with the full-length and two shorter constructs, IVP 4 and 5. Like the group I sera, group II sera uniformly failed to react with bacterial FPs in Western blots (Fig. 6 *A*). The third group of lupus sera represented by P.T., A.K., and Y.O. immunoprecipitated full-length protein (IVP 1) and degradation products, but they did not immunoprecipitate any of the COOH-terminal deleted translation products (Fig. 6 *C*). In contrast to groups 1 and 2, these sera were highly reactive in Western blots with the NH_2-terminal deletion FPs (FPs 1–4) and had variable reactivity with the other FPs, 8, 9, and 13 (Fig. 6 *B*). None of the group III sera reacted with FP 11, residues 4–68 (Fig. 6 *B*, and Table 2). An example of reactivity of group III sera is depicted in Fig. 6 *B* for A.K. serum.

Finally, there were two sera that appeared to combine the features of capability to immunoprecipitate multiple translation products as well as immunoblot FPs. Serum N.E., classified as SLE IVA, immunoprecipitated IVPs 1–3, but were unable to react with IVPs 4, 5, and 6 (Fig. 5 *D*). In Western blotting, this serum was reactive with all FPs, except for FPs 11 and 13 (Fig. 6 *C*). It was noted that serum N.E. reacted with FP-9 (residues 4–128) but did not precipitate IVP 4 (residues 1–128). Serum F.L., classified as SLE IVB, precipitated the full-length construct and IVPs 4 and 5 (Fig. 5 *E*), and in Western blots reacted with all FPs except FP 11 (residues 4–67), as depicted in Fig. 6 *D*. The background staining depicted in Fig. 6, *C* and *D* was high and could not be removed by repeated adsorptions with nontransformed *E. coli* lysates. However, repeat experiments confirmed the above findings with the sera.

Discussion

We have developed three sets of reagents for examination of the immunologically reactive structures of PCNA: cDNA-derived in vitro translation products, bacterial FPs, and a panel of synthetic 15-mer peptides. Application of these reagents using the complementary techniques of immunoprecipitation, immunoblotting, and ELISA has allowed us to probe the reactivities of both primary and tertiary structure-dependent epitopes. This approach was validated using the rabbit anti-peptide antibody raised against residues 11–23 of PCNA. By subtraction analysis of the results obtained using cDNA-derived IVPs and FPs, it was possible to map the RAPAb epitope to a region between residues 4 and 38 of PCNA, which conformed to the result obtained by ELISA. Similarly, the reactivity of mAb 19A2 was localized to a region between residues 68 and 128, consistent with previous *S. aureus* V8-induced proteolytic peptide mapping studies. The epitope was narrowed down to region 111–125 of PCNA by ELISA using synthetic peptides. These results demonstrate both the resolution of this approach and the sensitivity of the method for detection of primary sequence epitopes.

Several interesting observations emerge with analysis of the reactivity of lupus sera. A substantial number (9 of 14) did not blot recombinant FPs, this feature being characteristic of sera in groups I and II. Because sera in groups II, III, IVA, and IVB showed different reactivity with certain defined subfragments, it was possible to construct an epitope map by subtraction analysis, as shown in Fig. 7. Three sera in group II (Fig. 7, *top left*) were able to immunoprecipitate IVP5 (1–85) but not IVP6 (1–67), indicating that the sequence between residues 67 and 85 is an important component of the epitope. IVP4 retained reactivity, as did full-length PCNA, but

surprisingly, IVPs 2 and 3 were not reactive even though IVPs 4 and 5 are a subset of sequences present in the larger proteins. This implies that the additional residues in IVPs 2 and 3 interfere with the reactivity of the region 67–85. Reactivity, however, is restored in full-length PCNA (IVP-1), implying two possibilities. One is that the additional sequence at the COOH terminus in some way counteracts the interference with the reactivity of region 67–85 seen with IVPs 2 and 3. The other is that there may be a second antibody population that is dependent on the presence of the COOH terminus. Serum C.R. in this group showed a reactivity that could be explained by recognition of an epitope bound by residues 86–128, with no dependence on the COOH-terminal 212–261 peptide, since it is reactive with the constructs IVPs II and III lacking this region.

The sera in group III (Fig. 7, *top right*) reacted only in Western blots and were not found to immunoprecipitate less than full-length translation products. Sera A.K. and Y.O. blotted all NH_2-terminal deletion products to FP 4, demonstrating that the epitope was present downstream from residue 99. The smallest COOH-terminal deletion that retained reactivity extended to residue 128. Thus, the boundaries of the epitope must lie between residues 99 and 128. Serum PT would appear to require additional residues, extending its epitope from 99 to 150. These sera were unable to immunoprecipitate COOH-terminal deletion products even though these polypeptides possessed the sequences shown by Western blot to be required for immunoreactivity. This suggests that the epitope is sensitive to conformational changes induced by these restriction enzyme–engineered deletions. However, the spontaneous degradation fragments of full-length PCNA (IVP-F) are precipitated by these sera. It has been observed that proteolytic enzymes cleave between structured domains separated by flexible polypeptide fragments (25, 26). This might explain features observed by us previously that proteolytic digestion of nuclear antigens such as SS-B/La and PCNA result in degradation fragments, many of which retained reactivity with autoantibodies (18, 27). In the case of SS-B antigen, digestion with *S. aureus* V8 resulted in a fragment of 28 kD, which retained reactivity with autoantibody as well as RNA-binding property (28). It is obvious that PCNA fragments generated by the use of restriction enzyme cleavage of cDNA result in truncated protein products that do not correspond to the natural structural domains of PCNA.

The sera in groups IVA and IVB appeared to combine the properties of sera in group II (for immunoprecipitation) and group III (for Western blotting). Serum N.E., classified as group IVA (Fig. 7, *bottom left*), was reactive in Western blots like the group III sera, recognizing the peptide sequence 99–128. In immunoprecipitation, it appeared to require the region 128–150 to express the epitope. Serum F.L. of group IVB (Fig. 7, *bottom right*) was similar to group III and group IVA serum in Western blotting reactivity, but for immunoprecipitation, residues 68–128 and COOH-terminal 212–261 were required like the group II sera.

The differences between experimentally induced antibodies to PCNA and human autoantibodies were striking. Rabbit antipeptide antibody and monoclonal 19A2 were reactive with

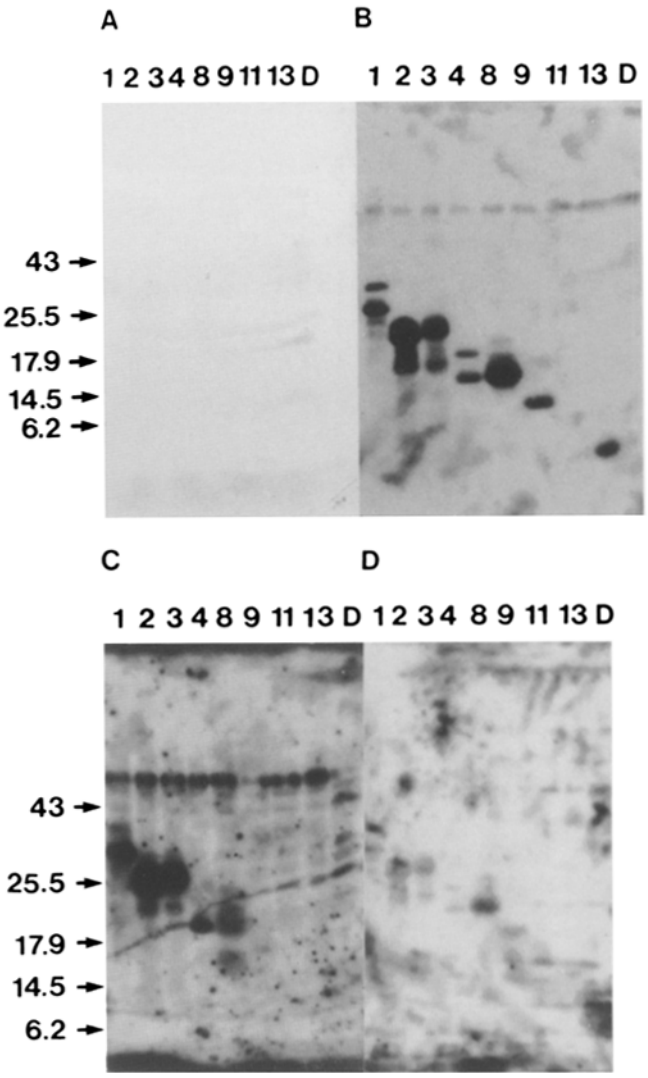

Figure 6. Western blots of FPs. Absence of reactivity with FP fragments from group II SLE sera is demonstrated in *A*. The reactivity of SLE group III sera (AK) is shown in *B*; group IVA (NE) in *C*; and group IVB (FL) in frame *D*. Nontransformed DH1 *E. coli* lysate is shown in lane *D*. The high backgrounds in *C* and *D* were characteristic of the sera and could not be removed with repeated absorptions with nontransformed *E. coli* lysate.

synthetic peptides, and in a consistent manner with defined subfragments of PCNA in immunoprecipitation and Western blotting. This is in marked contrast to lupus autoantibodies, which were completely nonreactive with synthetic peptides. The peptides were linear sequences of 15 residues, but with amino acid sequences overlapping in such a manner that any combination of six continuous residues in PCNA would be represented in this battery of synthetic peptides. The absence of reactivity indicates that the epitopes recognized by lupus sera are not present in these peptides. This observation coupled with the inability of the majority (9/14) of lupus sera to immunoblot recombinant FPs suggest that the epitopes recognized by certain human autoantibodies are comprised of higher ordered conformational structures not represented

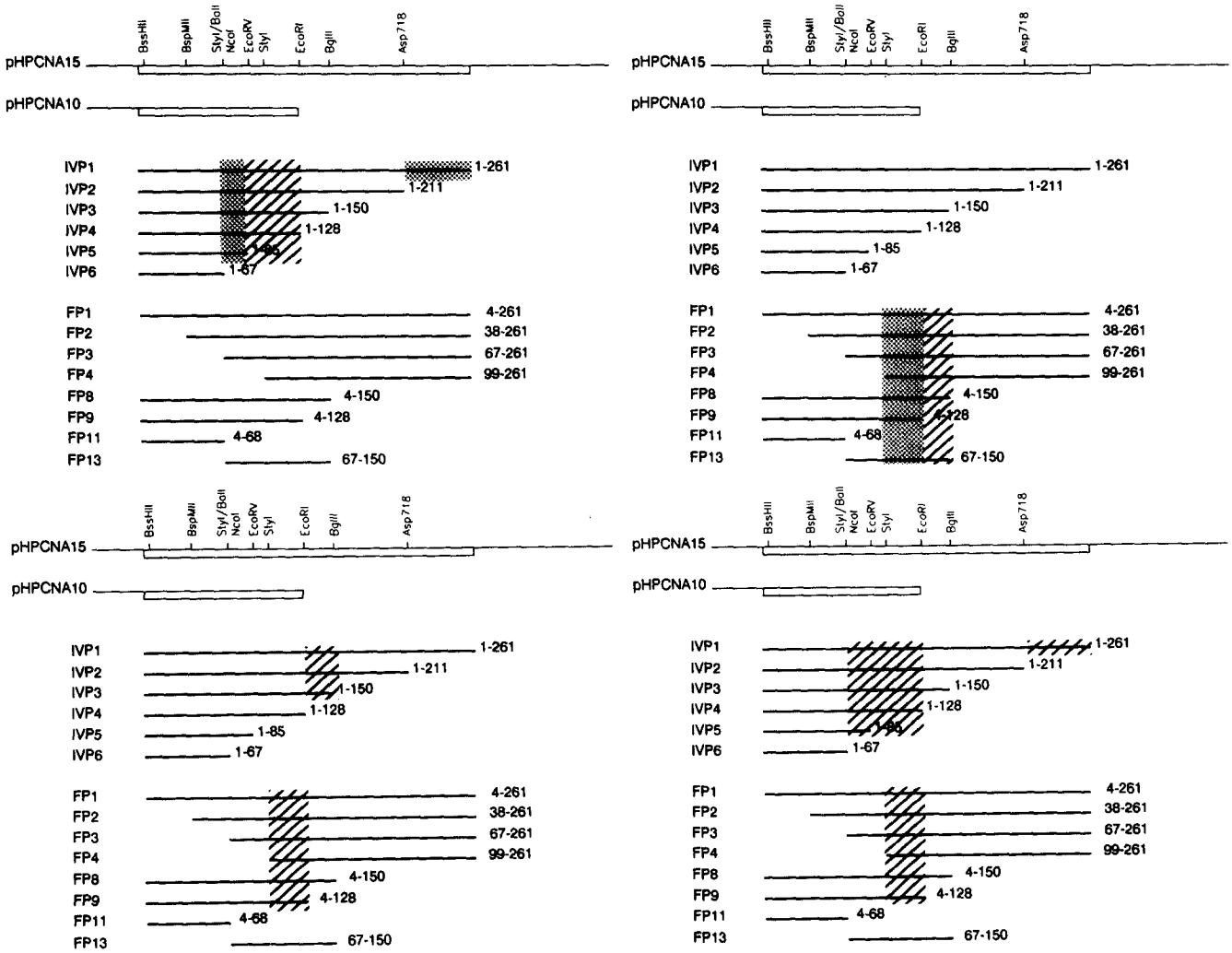

Figure 7. Epitope mapping of reactive regions in PCNA protein deduced by subtraction analysis of data derived from immunoprecipitation and Western blotting. SLE group II (*top left*) sera were reactive only by immunoprecipitation, and the regions necessary for reactivity could be localized to the stippled area for O.K., C.A., and E.B., and the hatched area for serum C.R. SLE group III (*top right*) sera were nonreactive with truncated translation products, but were reactive with FPs, and the regions necessary for reactivity were mapped to the shaded areas. The stippled area represented the reactivity of sera A.K. and Y.O. (*Bottom left*) The reactions with translation products as well as FPs deduced to be reactive with SLE group IVA (NE). (*Bottom right*) The reactivity of group IVB serum (FL).

in the synthetic peptides. This type of conformational structure may be formed by amino acid residues from two or more regions of the molecule that are brought into proximity with one another by protein folding. Geysen et al. (29) have analyzed the reactivity of a mAb directed against a discontinuous antigenic determinant in foot and mouth disease virus. They showed that a linear hexapeptide with the appropriate stereochemistry can mimic an epitope that was deduced to be derived from three separate regions of the antigen. It is possible that none of the peptides of PCNA that were synthesized contain the correct sequence, if the epitope was of such a nature.

The differences observed between mAbs and human autoantibodies to PCNA were not restricted to the mAb 19A2 reported here. In separate studies where reactivity with synthetic peptides in ELISA was the only assay examined, a second mAb, 19F4, was also reactive with these synthetic peptides. Differences between certain experimentally induced antibodies and spontaneously occurring human autoantibodies have also been demonstrated in a study in which five murine mAbs to SS-B/La, another nuclear autoantigen in lupus, were compared with spontaneously occurring autoantibodies from patients (30). It was observed that the murine mAbs had different reaction patterns from human autoantibodies, including the observation that autoantibodies were reacting with epitopes that were more highly conserved across species, while the monoclonals were restricted in their crossreactivity. It might be important to consider that although the immune systems in rabbits and mice (for experimental antibodies), and in patients (for autoantibodies), are responding to the same immunogen, the immunogen could be presented in different structural forms. It is unlikely that the patient's immune system

is encountering purified PCNA protein by itself, as in experimental immunization. There is much evidence that many of the intranuclear antigens are subcomponents of multimolecular particles (3, 31), and it is likely that the immune system in patients is encountering PCNA in the context of its association with other proteins or nucleic acids. The immunogen in patients appears to be subcellular particles, many of which are engaged in important or essential cellular functions (3, 32–38). Some observations indicate that epitopes of autoantigens in these particles might represent the active sites or functional regions, since autoantibodies are capable of inhibiting function, as in the case of PCNA (12–14), threonyl-tRNA synthetase (33), and other intracellular antigens (34–36, 38).

Autoantibodies to nuclear antigens have proved to be valuable reagents for cloning of DNA that encode these antigens. As a result, many cDNA clones have been generated, and a major direction of research in the field has been to make use of these clones and their protein products to identify the epitopes recognized by autoantibodies. This type of "epitope mapping" has been performed for centromere protein B (39), an autoantigen in patients with a subset of scleroderma, the A protein of small nuclear ribonucleoprotein particles (40), and a 68/70-kD protein that is an autoantigen in patients with mixed connective tissue disease (40–42), the SS-B/La antigen in patients with Sjogren's syndrome and lupus (43–46), the Ku or p70 DNA binding protein antigen in patients with connective tissue diseases (47), the Sm-B'/B antigen of small nuclear RNP particles (48), and histidyl-tRNA synthetase (49). In most of these studies, epitopes have been mapped with the use of recombinant fusion proteins in Western blotting, and there have been no studies using immunoprecipitation or comparison between human autoantibodies and experimentally induced antibodies. Although some have proposed that their data suggest an extremely high degree of heterogeneity and multiplicity of epitopes recognized by human autoantibodies, others have interpreted their data to mean that there might be one or two dominant epitopes with other minor epitopes. In the case of antigens such as SS-B, CENP-B, 68/70-kD protein, and the Sm B'/B antigen, some authors have also advanced the suggestion that epitopes might consist of noncontiguous sequences (39, 44–46, 48). An important issue in analysis of data obtained solely on the basis of immunoblotting is the possibility that the reaction with polypeptide bands may represent antibodies binding with partial epitopes. These partial epitopes might be segments of discontinuous regions that are brought into proximity by protein folding or by association of the antigen with other molecules in the form of intracellular particles. These issues could be clarified by elucidation of the three-dimensional structure of autoantigens, but until such knowledge becomes available, definition of epitopes recognized by autoantibodies would need to be interpreted with caution and should not be based on a single method of immunological analysis.

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