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Demonstration of Extrinsic DNA from Immune Complexes in Plasma of a Patient with Systemic Lupus Erythematosus

KUNIHIKO TERADA, EIJI OKUHARA*, YOSHIHIKO KAWARADA and SUSUMU HIROSE+

Department of Biochemistry, Akita University School of Medicine, 1-1-1 Hondo, Akita 010, and +National Institute of Genetics, 1111 Yata, Mishima 411, Japan

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Summary: Antigen DNA isolated from immune complexes present in plasma of three patients with active systemic lupus erythematosus using an affinity column was cloned and sequenced. One clone, designated pKS7, was found to have a region homologous with that of the E. coli metK gene, and another, designated pKS8, had a region homologous with a sequence including the replication origin of bacteriophage f1. Gel retardation assay revealed that pKS7 and pKS8 interacted with the patient's IgG fraction to form immune complexes, respectively. The affinity-purified antigen DNA was proved to be originated from bacteria or bacteriophage.

Autoantibodies against DNA in serum of patients with systemic lupus erythematosus (SLE) are a significant diagnostic marker (1). The failure of many investigators to induce the anti-dsDNA autoantibodies prominent in SLE in animal models (2-5) has led to the conclusion that the production of such antibodies in SLE occurs by some mechanism other than direct antigen stimulation (6). It is generally considered that antigen DNA is present in plasma of SLE patients in the form of so-called immune complexes, and that the manifestation of the clinical symptoms of SLE is closely associated with the occurrence of immune

* To whom all correspondence should be addressed.

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complexes in the circulating blood of affected patients (1,7,8). If so, it is necessary to separate the antigen DNA from the immune complexes in order to clarify the pathogenesis. Sano and Morimoto (9,10) and Van Helden (11) both isolated antigen DNA from sera of SLE patients. From the sequences of cloned antigen DNA, they considered that regions containing GC had greater potential for the formation of anti-Z-DNA antibodies in SLE patients.

Based on an assumption that some form of extrinsic DNA might be involved in the production of anti-dsDNA autoantibodies, we isolated antigen DNA from immune complexes in the circulating blood of patients with active SLE using an affinity column. The antigen DNA thus obtained was cloned and analyzed in order to examine its origin, and subsequently it was found to be derived from a bacterial or bacteriophage source, and not a human one.

Materials and Methods

Blood Heparinized blood from patients with SLE was kindly provided by Dr. T. Yamagishi, the Japan Red Cross Hospital, Akita, and Dr. A. Kuwayama, the Yuri Union General Hospital, Honjo city, Akita. After the blood had been transferred to the laboratory, anti-DNA antibody titers were examined by an improved ELISA devised in our laboratory (12). The plasma from each of three patients described in this paper was positive for anti-DNA autoantibody. Healthy human blood was donated by students at the School of Nursing, Akita University.

Separation of plasma Ten milliliters of each blood sample was immediately diluted with an equal volume of equilibrated salt solution (0.126 M NaCl/5.0 x 10^{-6} M $CaCl_2$/9.8 x 10^{-5} M $MgCl_2$/0.01% anhydrous D-glucose/0.014 M Tris-HCl, pH 7.6), and then carefully layered upon 20 ml of Ficoll-Paque. After centrifugation (400 x g, 20°C, 30 min), the upper layer (plasma) was saved.

Preparation of DNA Plasma was applied to a column of anti-human IgG antibody-bound Sepharose 4B prepared according to the method of Kitagawa and Okuhara (13). After washing with phosphate-buffered saline (PBS), 1 M NaCl and PBS in order,

DNA was eluted with 20 mM $NaHCO_3$, pH 10.5/5% dimethyl sulfoxide (DMSO). The eluate was applied to a DEAE-cellulose column, and then the DNA fraction was eluted with 2 M NaCl. This fraction was treated with RNase, phenol and then ether. The DNA solution was desalted and concentrated by using a NENSORB-20 column (NEN, USA). The final eluate with 50% methanol was evaporated under a nitrogen stream and stored at -20° C. DNA of normal individuals was prepared in the same manner as in patients' DNA.

Preparation of IgG After elution of antigen DNA from the column, the column was washed with PBS and the IgG fraction was eluted with 4 M guanidine-HCl. The eluate was dialyzed against PBS and the resulting precipitate was removed by the centrifugation. NaN_3 was added to the supernatant solution to a concentration of 0.02%, and the solution was stored at 4° C.

Cloning and sequencing of antigen DNA DNA from a SLE patient, K.S., was blunt-ended with T_4 DNA polymerase and ligated into pUC18 with T_4 DNA ligase. The ligated DNA was transformed into *E. coli* HB101, and from the large number of recombinant clones, nine were selected. The nucleotide sequence of the insert DNA was determined by the dideoxy method (14,15) and the sequence homology was analyzed using a Gene-Master™ (Bio Rad, USA).

Southern hybridization Southern hybridization analysis was carried out according to Southern (16) with minor modifications. DNA was subjected to 1% agarose gel electrophoresis and then transferred to a nylon membrane (Gene Screen Plus™, NEN, USA). After UV irradiation for 1 h, the membrane was prehybridized in 10 ml of a solution containing 50% formamide/1 M NaCl/1% sodium dodecyl sulfate (SDS) at 42° C for 15 min. Subsequently, thermally denatured ^{32}P-labeled probe DNA and 100 μg salmon sperm DNA in 1 ml were added to the solution and hybridization was carried out at 42° C for 20 h. After hybridization, the membrane was washed twice in 2 x SSC/0.1% SDS at room temperature for 5 min, then once in 1 x SSC/0.1% SDS at 42° C for 1 h and twice in 0.1 x SSC/0.1% SDS at 42° C for 30 min. Autoradiography was performed at -80° C for 5 days or longer. The probe DNA was prepared as follows; DNA was cleaved from recombinant clones with BamHI and KpnI, and then labeled with [α-^{32}P] dCTP (111 TBq/mmol, ICN, USA) according to the random-primed labeling method (17,18). The specific activity of the probe DNA was $0.5 \sim 1.0 \times 10^8$ cpm/μg.

Gel retardation assay (19-21) DNA was labeled with ^{32}P at the 5' termini, and IgG was labeled with ^{125}I using IODO-GEN® reagent (PIERCE, USA). The mixtures of DNA and IgG in 20 μl of a solution containing 10 mM NaH_2PO_4, pH 7.2/150 mM NaCl were incubated on ice for 1 h and electrophoresis was run on 4.5% polyacrylamide gel in 0.5 x Tris-borate buffer at 4° C at 30 mA until free DNA ran out. The gel was dried *in vacuo* and autoradiographed at -80° C using intensifying screens.

Results and Discussion

Fig. 1 shows the base sequences of two clones, pKS7 and pKS8. We searched for homology sequences in the data bank, and found that one clone, pKS7, had 92% homology with the 3'-untranslated region of the E. coli metK gene, which encodes S-adenosyl methionine synthetase (Fig. 1A), and another, pKS8, had 94% homology with a sequence including the replication origin of bacteriophage f1 (Fig. 1B).

In order to verify whether the cloned DNA was present in plasma of SLE patients, 100 ng of each DNA from three patients as well as that from seven healthy controls was subjected to 1% agarose gel electrophoresis, transferred to a nylon membrane and hybridized with radioactive cloned DNA.

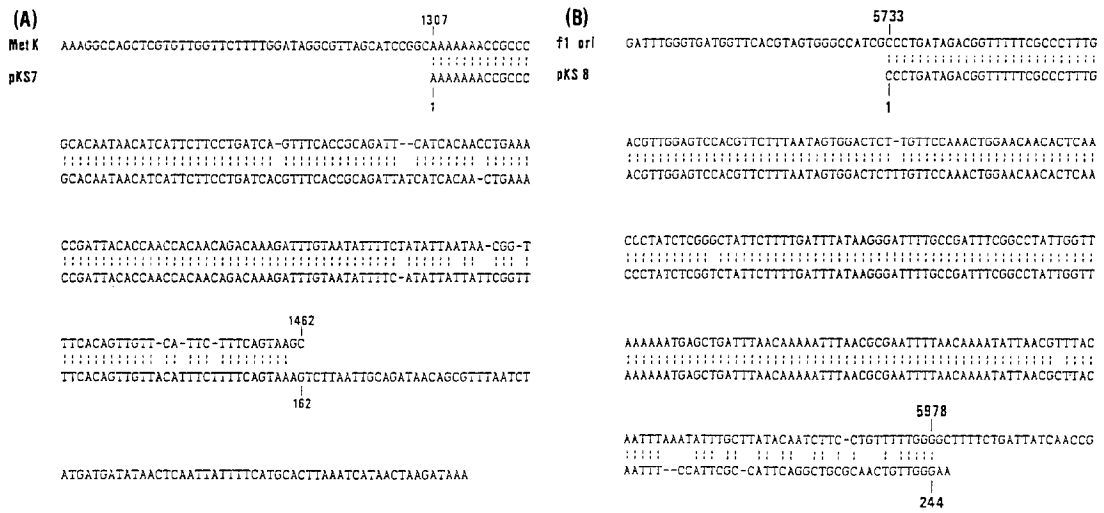

Fig. 1. Sequence comparisons of pKS7 with E. coli metK and pKS8 with bacteriophage f1.

A: Aligned sequences of pKS7 and E. coli metK. The region from 1307 to 1462 in the nucleotide sequence of metK has 92% homology with the region from 1 to 162 in the nucleotide sequence of pKS7 (number of nucleotides of metK registered in the data bank is 1462.) B: Aligned sequences of pKS8 and bacteriophage f1. The region from 5733 to 5978 in the nucleotide sequence of bacteriophage f1 has 94% homology with the region from 1 to 244 in the nucleotide sequence of pKS8.

pKS stands for each clone, together with an individual number.

As shown in Fig. 2, both pKS7 and pKS8 hybridized with the DNAs from the three SLE patients, but not with the control DNAs. Moreover, when other E. coli chromosomal DNAs such as rpsA, recA, and dnaQ were used as probes, none of these probes hybridized with the antigen DNA (data not shown).

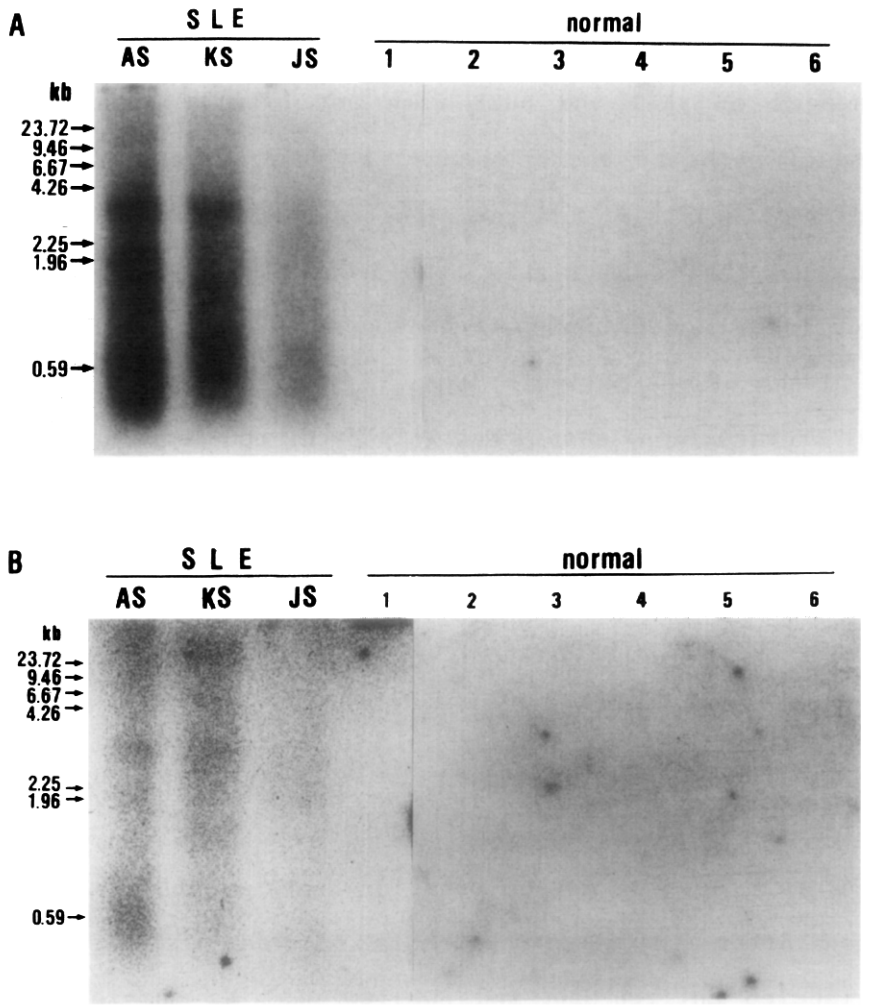

Fig. 2. Hybridization of antigen DNA in patients' plasma with cloned DNA.

Each of the DNA fragments from three SLE patients (A.S, K.S and J.S.) and seven normal individuals was subjected to 1% agarose gel electrophoresis and then transferred to a nylon membrane and hybridized with (A) pKS8 (0.5 x 10^8 cpm/μg) or (B) pKS7 (1.0 x 10^8 cpm/μg). Autoradiography was performed at -80°C for 8 days for pKS8 and for 5 days for pKS7. The amount of DNA used for the electrophoresis was 100 ng.

Hereupon, it comes into question whether contamination with E. coli or bacteriophage during the preparation of antigen DNA had taken place or not. However, it can be denied from the experimental results described above, that is, 1) both pKS7 and pKS8 did not hybridize with seven control DNAs which were prepared in the same manner as in the patients' DNA. 2) Other E. coli chromosomal DNAs such as rpsA, recA, and dnaQ did not hybridize with the antigen DNA. 3) The sequences of pKS7 and pKS8 were not exactly identical to those of metK and bacteriophage f1, respectively, which are used routinely in laboratories. Accordingly, it can be concluded that DNAs highly homologous with portions of the E. coli or bacteriophage f1 genome were actually present in the plasma of SLE patients.

Gel retardation assay was employed to demonstrate the formation of DNA-antibody complexes. One nanogram of each labeled DNA fragment and 10 μg of IgG were mixed together and electrophoresed on 4.5% polyacrylamide gel. As seen in Fig. 3, the slow moving bands on lanes 2 and 5 were found to be located at the position of the slowest moving band of ^{125}I-labeled SLE-IgG (lane 1). On the other hand, ^{32}P-labeled antigen DNA moved until the end of the gel. Therefore, the slow moving bands on lanes 2 and 5, which appeared after the electrophoresis of the mixtures having ^{32}P-labeled antigen DNA and SLE-IgG must be complexes produced between KS7 or KS8 and SLE-IgG. As a result, the data proved that antigen DNA, KS7 and KS8, formed complexes with SLE-IgG, respectively.

In conclusion, we have proved that the affinity-purified DNA from SLE patients originates from bacteria or

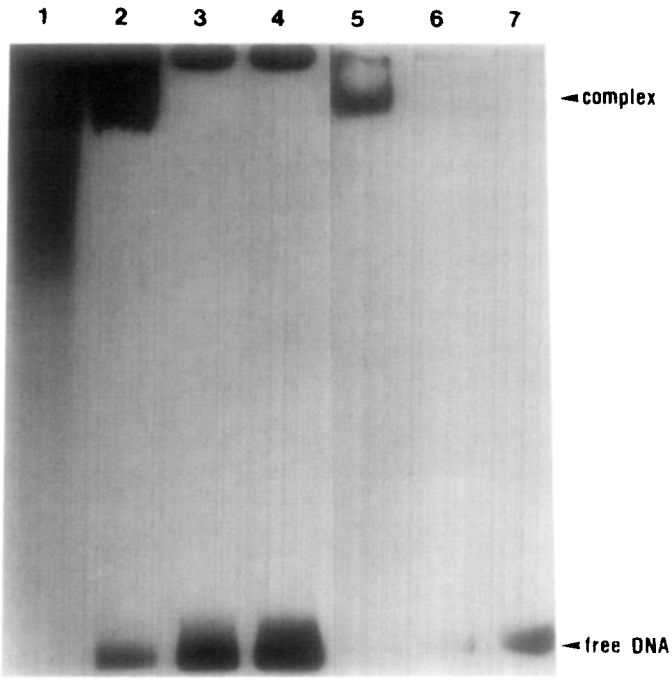

Fig. 3. Binding ability of antigen DNA to specific anti-DNA antibodies in IgG fraction.

One ng of 5'-^{32}P-labeled DNA fragment was incubated with 10 μg of IgG fraction prepared from a SLE patient or a normal individual, and applied to 4.5% polyacrylamide gel electrophoresis in 0.5 x Tris-borate buffer with constant circulation at 4°C. The gel was dried *in vacuo* and autoradiographed at -80°C for 2 days. lane 1, ^{125}I-SLE IgG; lane 2, ^{32}P-pKS7-243 bp + SLE IgG; lane 3, ^{32}P-pKS7-243 bp + normal IgG; lane 4, ^{32}P-pKS7-243 bp; lane 5, ^{32}P-pKS8-246 bp + SLE IgG; lane 6, ^{32}P-pKS8-246 bp + normal IgG; lane 7, ^{32}P-pKS8-246 bp.

bacteriophage using molecular cloning techniques. The present study supports the notion that bacterial or viral infection is closely related to the onset of SLE (22,23).

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