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Deoxyribonucleic acid strandedness. Partial characterization of the antigenic regions binding antibodies in lupus erythematosus serum.

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Research Article

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Deoxyribonucleic Acid Strandedness

PARTIAL CHARACTERIZATION OF THE ANTIGENIC REGIONS BINDING ANTIBODIES IN LUPUS ERYTHEMATOSUS SERUM

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ABSTRACT This study shows that tritiated thymidine labeled DNA prepared from mammalian cells by the Marmur technique is a pure preparation of nucleic acid that is composed essentially of two populations of molecules. One molecular population consists of primarily double-stranded nucleic acid, while the other population is of double-stranded nucleic acid with significant single-stranded regions. The double-stranded DNA with single-stranded regions can, depending upon the length of the single strand, behave as "native" DNA or "denatured" DNA on methylated albumin kieselguhr (MAK) column chromatography. Using MAK chromatography we have separated the DNA into a salt-elutable fraction composed of primarily double-stranded molecules and an alkaline-elutable fraction containing double-stranded nucleic acid with variable length, single-stranded regions. Endonuclease enzyme removal of the single-stranded regions from the alkaline fraction DNA yields nucleic acid that behaves identically to the salt elutable DNA. Exonuclease removal of the single-stranded regions suggests they are located primarily at the ends of the molecules. Our data show that the alkaline-elutable DNA differs from salt-elutable DNA only in that the former has significant single-stranded regions.

Sera of patients with systemic lupus erythematosus (SLE) selected for anti-DNA by hemagglutination bind significantly less to the alkaline fraction DNA than the salt fraction DNA. This difference in binding clearly does not represent simply an affinity for double-stranded vs. single-stranded nucleic acid since the alkaline fraction DNA contains predominately double-stranded nucleic acid. A model for antibody-DNA binding is suggested from the present data and information contained in the literature.

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INTRODUCTION

Antibodies to DNA occur in a variety of disease states (1). However, antibodies to native DNA are felt by many to occur almost exclusively in systemic lupus erythematosus (SLE)[1] (1-4). In contrast, antibodies to native DNA have been reported in 19% of patients with rheumatoid arthritis, in 24% of patients with primary and secondary uveitis, and even in normal sera (5-7). While it has been shown that experimentally induced anti-DNA antibodies react only with denatured DNA, most spontaneous antibodies from patients with SLE react with both denatured and native DNA (1, 2, 8, 9). The occurrence of antibodies against native DNA is used both diagnostically and prognostically even though there is difficulty in separating reactivity of the antibodies with denatured DNA from that with native DNA (10, 11). Thus, there is some question of the frequency of occurrence of antinative DNA antibodies and the immunologic specificity of their reaction with DNA.

In probing the nature of the antibodies to DNA in the disease SLE and related diseases, we feel it imperative to be able to define the structure of the nucleic acid used in the various antigen-antibody tests. To better define the nature of purified mammalian DNA used in DNA-antibody binding tests, we have characterized the structure of this DNA by chromatographic and centrifugation techniques before and after enzyme removal of single-stranded re-

[1] *Abbreviations used in this paper:* A-T, adenine-thymine; G-C, guanine-cytosine; KB, human epidermoid cancer cells; MAK, methylated albumin kieselguhr; Scintisol, Scintisol-Complete; SLE, systemic lupus erythematosus.

gions contained in the nucleic acid molecules. We have found that purified mammalian DNA is a heterogeneous mixture of nearly homogeneously double-stranded DNA and double-stranded DNA with significant single-stranded regions. The double-stranded DNA with significant single-stranded regions can, depending upon the length of the single strand, behave as "native" DNA or "denatured" DNA in the commonly utilized chromatographic and centrifugation techniques.

In this report, we have detailed our experimental approach to the characterization of subtle structural defects in the molecules of purified DNA. We have examined in more detail the structural aspects of purified mammalian DNA since this nucleic acid is commonly used in studying the antibodies to DNA found in the sera of patients with SLE. Our studies show that structural defects in the purified DNA molecule profoundly influence the binding of nucleic acid to the antibodies in serum of patients with SLE.

METHODS

DNA preparation. Human epidermoid cancer cells (KB-A 2120) obtained from Dr. Andrew M. Lewis, Jr. of the National Institute of Allergy and Infectious Diseases, National Institutes of Health were grown in spinner culture in a MEM Spinner medium (Grand Island Biological Co., Grand Island, New York) to a cell density of 500,000 cells per ml. The cells were then diluted with fresh medium to a density of 200–250,000 cells per ml, and 0.3–1.0 mCi of tritiated thymidine (New England Nuclear, Boston, Mass.) was added in a total 1,000-ml culture. After incubation for 24 h at 37°C, the culture was harvested by centrifugation and washing with two changes of saline. The cells were kept frozen at −20°C until isolation of the DNA by the Marmur method (12).

Calf thymus DNA (Sigma Chemical Co., St. Louis, Mo.) was purchased from Sigma as the type I highly polymerized sodium salt. DNA concentrations were measured by the diphenylamine procedure of Burton (13), and protein concentration was determined by the technique of Lowry, Rosenbrough, Farr, and Randall (14). For each determination, a standard curve using calf thymus DNA or bovine albumin (Sigma Chemical Co.) was prepared. It has previously been shown that the Lowry test with bovine albumin as a standard is an adequate measure of histone (3).

Methylated albumin kieselguhr chromatography. Methylated albumin kieselguhr (MAK) chromatographic fractionation was performed after the technique of Mandell and Hershey (15) with modifications suggested by Sueoka and Cheng (16). 10 g of kieselguhr (Johns Manville Co., Maryland Heights, Mo.) was brought to a boil in 50 ml of 0.1 M NaCl-0.05 M sodium phosphate buffer, pH 6.7 (NaCl buffer), and allowed to cool. 2.5 ml of a 1% solution of methylated albumin (Sigma Chemical Co.) was added to the kieselguhr stirring at room temperature. The final solution was stirred at low speed for 30 min and repeatedly poured and packed by air pressure into a chromatographic tube (Pharmacia Fine Chemicals Inc., Piscataway, N. J.). Finally, 1 cm of a boiled and cooled solution of 0.5-g kieselguhr in 10 ml NaCl buffer was packed on top. The columns were then washed with 15 vol of NaCl buffer, stored at room temperature, and used within 7 days. For each group of columns, a previously chromatographed DNA preparation was chromatographed on one column as a test of the MAK preparation.

Elution of DNA on MAK columns. The nucleic acid sample was added to the column in a solution of NaCl buffer at a concentration not exceeding 100 μg DNA/ml buffer, and the flow was subsequently maintained at 0.85 ml/min by a peristaltic pump (LKB Instruments Inc., Rockville, Md.). The column was then washed with 5–10 column volumes of NaCl buffer. Salt gradient elution was accomplished by linear or stepwise increasing gradients of NaCl in 0.05 M sodium phosphate buffer, pH 6.7. The gradients were generated by a Buchler rectangular varigrad (Buchler Instruments Co., Fort Lee, N. J.). After the salt elution, the remaining column bound DNA was recovered by a stepwise elution with 1.0 M NaCl–0.05 M sodium phosphate buffered to different alkaline pH's. The salt gradients were monitored by refractive index on a Bausch and Lomb (Bausch and Lomb, Rochester, N. Y.) refractometer, and the pH of fractions were monitored by a pH meter (Corning Scientific Instruments, Medfield, Mass.). Chromatography was always carried out at room temperature. Aliquots of 2-ml fractions were mixed in 5–10 ml of Scintisol-Complete (Scintisol, Isolab Inc.. Akron, Ohio) and radioactivity counted in a Packard Tricarb liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.). Recovery of DNA from the column was usually 95–100% of the added radioactivity. MAK purified DNA had no detectable RNA and less than 1 μg/ml of protein. Sucrose centrifugation experiments indicate that the human epidermoid cancer cells (KB) DNA had an average molecular weight of about 10^6.

Cesium chloride equilibrium density centrifugation. Cesium chloride (CsCl, Harkshaw Chemical Co., Solon, Ohio) was dissolved in 0.01 M Tris-HCl buffer, pH 7.8, and the final density of the solution adjusted to approximately 1.715 g/ml by determining its refractive index in a refractometer (Bausch and Lomb Inc.). DNA samples were mixed in a total of 5.0 ml of CsCl solution and centrifuged immediately in Beckman polyallomer tubes (Beckman Instruments Inc., Fullerton, Calif.). The centrifuge tubes had been siliconized by coating the interior with a 100-fold dilution of Siliclad (Clay Adams Co., Parsippany, N. J.) and dried overnight at 60°C. Centrifugation was carried out by the fixed angle method of Flamm, Bond, and Burr (17) in a type 65 rotor of a Beckman L2-65B ultracentrifuge for 40 h at 40,000 rpm at 20°C to establish a stable concentration gradient. The contents of each tube were sampled by piercing the bottom of the tube and collecting 150 μl fractions using a constant volume displacement device (Micro-Metric Instruments Co., Cleveland, Ohio). The formation of the gradients were checked by measuring the refractive index of alternate fractions. Radioactive scintillation counting was performed on 25–50 μl aliquots dissolved in Scintisol.

Neurospora crassa endonuclease. The *Neurospora crassa* nuclease is an enzyme specific for single-stranded nucleic acid first described by Linn and Lehman (18, 19). The enzyme used in this study was purified from conidia purchased from Miles Laboratories Inc., Elkhart, Ind. (control no. 21-1-758) by the technique of Rabin, Preiss, and Fraser (20). For each enzyme isolation, 250 g of frozen conidia paste was thawed overnight at 0–4°C and suspended in 800 ml of 0.05 M glycylglycine buffer, pH 7.0. 50-ml aliquots in a beaker suspended in an ice acetone bath were sonicated for 6 min at full power with a Branson model 75 sonicator fitted with a flat tip on a disrupter horn (Ultrasonic Systems, Inc.. Farmingdale, N. Y.). Our

isolation procedure was modified according to suggestions of the Rabin et al. (20) method in that we purified the wash fraction obtained from the phosphocellulose chromatography. We did not include the Sephadex G-200 gel filtration step, but used the DEAE chromatography and final purification on hydroxyapatite.

The hydroxyapatite enzyme fraction was dialyzed against 0.02 M potassium phosphate, pH 6.5 and concentrated using a model 12 Amicon ultrafiltration cell fitted with a PM-10 filter (Amicon Corp., Lexington, Mass.). During use, the enzyme sample was kept at 0–4°C with no detectable loss in activity for at least 6 mo.

The final enzyme preparation had a sp act of 2,000 U/mg as measured by the technique of Rabin and Fraser (21). The enzyme is highly specific for single-stranded polynucleotides although nicks are introduced in double-stranded DNA upon prolonged incubation (20). To test the specificity of our enzyme preparation, a prolonged incubation of 10 U of the enzyme with 2 ml of 36 μg/ml of undenatured and heat-denatured tritiated thymidine-labeled *Escherichia coli* DNA was carried out.

After 27 h of incubation of undenatured DNA with enzyme, only 2% of input radioactive counts were made acid soluble. This apparent activity of the enzyme on native DNA is due to single-stranded material contaminating the nucleic acid preparation as shall be shown in subsequent experiments. Enzyme digestion of denatured DNA is complete after about 1 h of incubation under these conditions. The enzyme-resistent DNA in the denatured preparation is due to residual double-stranded DNA, as will be shown later in the paper.

Neurospora nuclease digestion of DNA with single strands. All enzyme incubation was carried out in 0.1 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$, and 0.1 M NaCl (22). If the DNA solution was concentrated, the incubation mixture containing enzyme was made up in a small test tube or vial which was then incubated in a water bath at 30°C. In some experiments the DNA solution was dialyzed in a stiff colloidon bag (Schleicher and Schuell Co., Keene, N. H.) against the above buffer, and then the enzyme was added. Incubation was then carried out in the dialysis tube suspended in buffer in a beaker in a water bath. The dialysis technique resulted in no loss of DNA via sticking to the membrane.

Exonuclease digestion of DNA. UV-exonuclease is an enzyme isolated from *Micrococcus luteus* which is associated with the repair of ultraviolet light-induced photoproducts in DNA (23). This enzyme is unique in that it will digest only the single-stranded ends of unirradiated DNA (24). It degrades the substrate ends from both the 3′ and 5′ termini in an exonucleolytic process yielding mononucleotides as its final product. The enzyme will not attack native DNA. The enzyme used in this study was a generous gift of Ms. Barbara Garvik and Dr. Lawrence Grossman, Department of Biochemistry, Brandeis University, Waltham, Mass.

The incubation mixture for the enzyme experiments contained 0.01 M Tris-HCl buffer pH 8.0, 0.005 M $MgCl_2$, 0.06 M KCl, and 0.001 M mercaptoethanol. Under these conditions, 1 U of enzyme will digest 1 μmol of DNA in 30 min at 37°C. The total volume, DNA concentration, and units of enzyme of the mix are described under Results.

Assay for release of radioactivity via enzyme digestion of nucleic acid. Aliquots for sampling were pipetted into a test tube at 0–4°C, and an equal volume of cold carrier calf thymus DNA (3.0 mg/ml in 0.015 M sodium citrate, pH 6.7) was added. To this solution was immediately added an equal volume of cold 10% trichloracetic acid, and the final mixture was allowed to stand in ice for 10 min. The acidified aliquots were then centrifuged at 13,000 *g* for 10 min at 4°C in a Sorvall RC2-B centrifuge (DuPont Instruments, Sorvall Operations, Newtown, Conn.). An aliquot of the centrifuged supernate was removed and dissolved in Scintisol for radioactive counting. The percent of nucleic acid radioactivity liberated was computed on the basis of counts made acid soluble per total input radioactivity. In all experiments, a control mixture with nucleic acid and no enzyme was included to measure background counts released during incubation.

DNA binding technique. The DNA binding test was a modification of the test described by Wold, Young, Tan, and Farr (25). Selected sera from normal hospital personnel and patients with SLE were separated within 1 h of drawing and stored at −20°C. Positive and negative sera were selected by results from our DNA hemagglutination test (3). The KB DNA (unfractionated and chromatographically fractionated) was kept frozen at −20°C or thawed once and stored at 4°C in an ice bucket. Before testing, unfractionated KB DNA was diluted with 0.05 M Tris-HCl buffer, pH 7.8, and the salt and alkaline fractions were dialyzed against the same buffer. DNA, buffer, and then serum were added, with mixing after each addition, to give a final reaction mixture volume of 100 μl. The tubes were incubated at 37°C for 1 h and then at 4°C for 18 h. An equal volume of saturated ammonium sulfate was then added and the tubes put at 4°C for 1 h with mixing every 15 min. The precipitate was pelleted by centrifugation at 1,000 *g* for 45 min in a PR-J centrifuge at 4°C. The precipitate was washed twice with 50% ammonium sulfate, dissolved in 0.5 ml of distilled water, transferred to scintillation vials, and 5 ml of Scintisol was added. The radioactivity was counted in a Packard liquid scintillation counter (Packard Instrument Co.) for a time sufficient to give 5% accuracy in counting. All determinations were made in duplicate.

RESULTS

MAK chromatographic fractionation of purified DNA. After binding to the column, native DNA can be eluted using a buffered salt gradient while denatured DNA or DNA containing single-stranded areas bind tightly to the column and may require a salt buffer with an alkaline pH before it can be eluted (26). The capacity of MAK columns is about 1 mg of DNA for 10 ml of MAK suspension (16).

A MAK profile of purified KB DNA was obtained by chromatographing 180 μg of KB DNA on a 10-ml MAK column and eluting first with a linear salt gradient followed by a stepwise increase in pH using three solutions of 1.0 M NaCl–0.05 M sodium phosphate buffered to pH 7.0, 10.7, and 11.6. The elution profile revealed a broad double-peaked salt elution which suggests heterogeneity of the "double-stranded" DNA, followed by three peaks in the alkaline gradient. The three fractions in Fig. 1 were pooled and the DNA and protein content measured. Fraction I contained 60 μg of DNA, Fraction II 72 μg, and Fraction III 36 μg. A total of 168 μg of DNA was recovered out of 180 μg added giv-

ing a minimum of 93% recovery since not all the fractions were measured for nucleic acid. All three fractions contained less than 1.0 μg of protein per ml, indicating that albumin was not stripped from the column with the alkaline solutions. When the fractions were rechromatographed on a MAK column, the salt-elutable fraction (I) contained radioactivity that eluted in the alkaline gradient, and, conversely, the alkaline-elutable fractions (II and III) contained radioactivity that now eluted in the salt gradient. It appeared, therefore, that the initial fractionation of relatively large quantities of DNA on the MAK columns was incomplete in that it did not strictly separate double-stranded molecules from molecules with single-stranded regions.

MAK chromatographic purification of salt-elutable and alkaline-elutable DNA. To prepare nucleic acid fractions of KB DNA that either eluted only in the salt or alkaline gradient, we decided to simplify the elution technique and employ rechromatography of the fractions. A total of 400 μg of KB DNA in 4 ml was passed onto a 20-ml MAK column, and elution was carried out with 5.0 M buffered salt solution followed by a 1.0 M alkaline (pH 11.6) buffered salt solution. The peak tubes of the salt and alkaline elution were pooled separately, dialyzed overnight against NaCl buffer, and rechromatographed. The process was repeated until the salt and alkaline fraction had been rechromatographed a total of three times. The final chromatography was carried out on a 2.5-ml MAK column to facilitate concentration of the nucleic acid. For each final fraction (salt or alkaline), about 95% of the input radioactive counts eluted in the salt or alkaline part of the chromatography (upper panel of Fig. 2). The two peak tubes from the final MAK purification were pooled and dialyzed against 0.05 M Tris-HCl, pH 7.8 (these will be designated salt and alkaline fraction in the

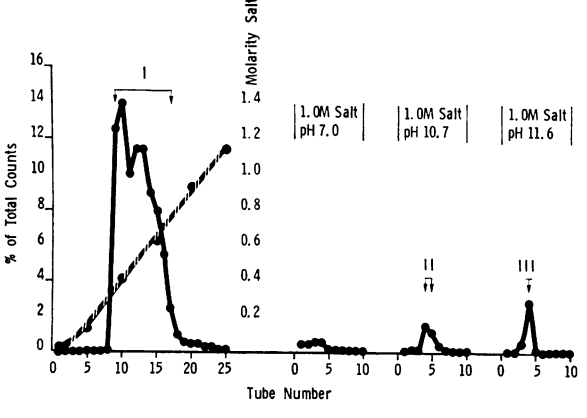

FIGURE 1 MAK purification of large quantities of KB DNA. Percent of radioactive counts (●——●). Salt gradient (●//●).

TABLE I

Recovery of DNA on MAK Columns of Salt/Alkaline Fractions before and after Removal of Single Strands

	Salt	Alkaline
Amount DNA added to column (before enzyme)	20 μg	20 μg
Amount DNA recovered from column (after enzyme)	20.5 μg	17.5 μg
Nucleic acid digested by enzyme	0%	12.5%
Counts made acid soluble by enzyme	1.6%	72%

experiments to be discussed). The DNA concentrations of the final salt and alkaline fractions were both about 40 μg/ml.

Enzyme treatment of MAK Salt and alkaline fractions. 200 μl of salt and alkaline fractions containing 8 μg of DNA were then tested for behavior on MAK columns before and after incubation with the single-strand specific endonuclease as shown in Fig. 2. Before enzyme treatment, greater than 90% of the radioactivity of either fraction eluted in the appropriate part of the eluant profile as shown in the upper section of Fig. 2.

500 μl of each fraction were dialyzed for 24 h against 1,000 ml of endonuclease buffer at 4°C with one change of buffer. The dialyzed solutions were added to capped vials along with 45 U (10 μl) of enzyme and incubated at 30°C for 20 h. Aliquots of the mixture were sampled for total and acid-soluble radioactive counts. The salt fraction released 1.6% of its radioactivity while the alkaline fraction released 72% of its radioactivity. The remaining portion of each fraction was then added to a 2.0-ml MAK column and chromatographed as described above. As shown in Fig. 2, the enzyme-treated fractions both eluted in the salt eluant with only about 5% of the radioactivity coming out in the alkaline eluant. Tubes two and three of each chromatographic profile were pooled and the DNA content measured. The results of the experiment are summarized in Table I. As might be expected, the salt fraction released about 1% of its radioactivity and lost a nonmeasurable amount of nucleic acid to enzyme incubation. It can be seen that about 10% of the alkaline fraction DNA was digested with the single-strand specific enzyme, and, after enzyme digestion of the single strands, the alkaline fraction DNA behaved like uniformly double-stranded DNA. This experiment was repeated once with the same results. Thus, enzyme treatment of the rechromatographed salt fraction resulted in a nonmeasurable amount of DNA released, 1.6% of radioactivity released,

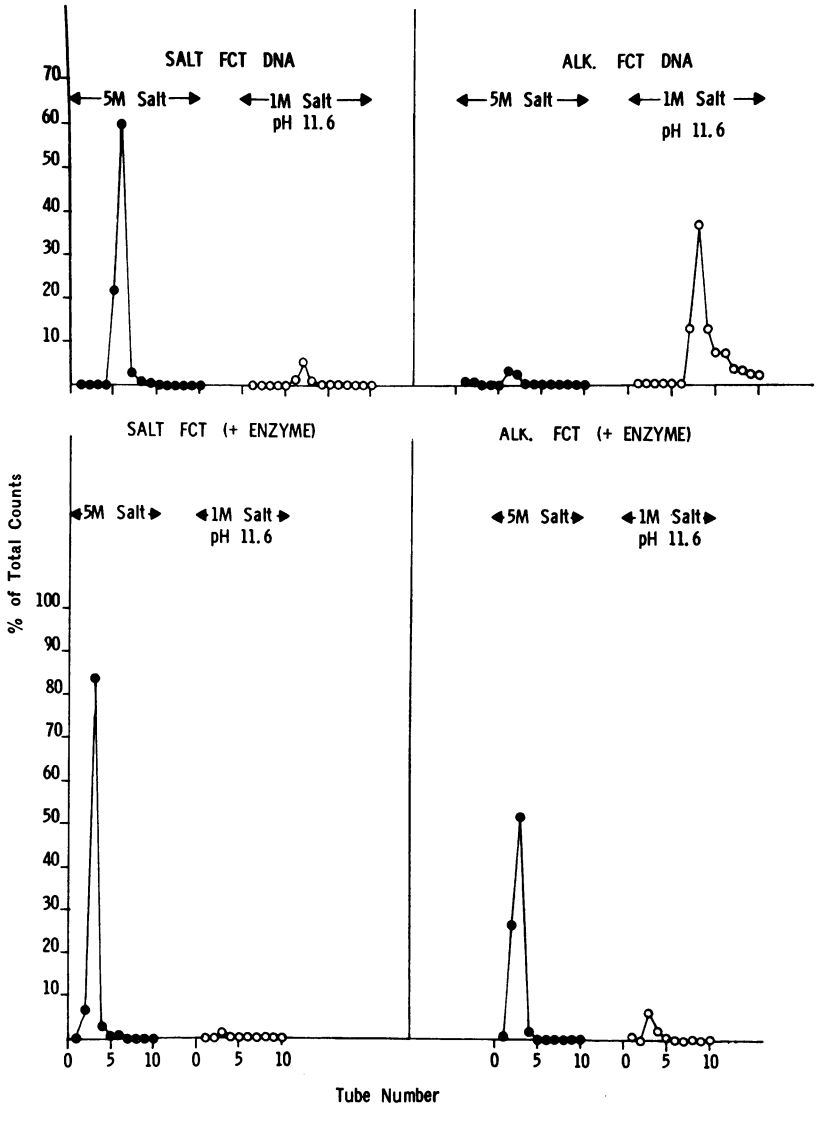

FIGURE 2 MAK profile of three times rechromatographed salt and alkaline fraction DNA before (upper panels) and after (lower panels) endonuclease removal of single strands. See Table I and text for quantitation.

and elution of the enzyme treated fraction over a more narrow salt molarity buffer. The alkaline fraction, on the other hand, released 72% of its radioactivity, 12.5% of its nucleic acid, and most of the enzyme resistant DNA eluted in the salt buffer.

The following experiment was done to demonstrate the specificity of the endonuclease. Rechromatographed enzyme-treated salt fraction containing approximately 0.5 μg of DNA was incubated with 45 U of endonuclease in 0.3 ml of buffer. This repeat incubation with a large amount of fresh enzyme (about a 40-fold increase in enzyme U/μg DNA) resulted in only about 0.04% of input radioactive counts being released above the control. This experiment emphasizes the specificity of the endonuclease for single-stranded nucleic acid only.

CsCl equilibrium density gradient centrifugation. The technique and interpretation of data gathered from CsCl equilibrium density gradient centrifugation has been well described by Szybalski and Szybalski (27). It has been shown that one can calculate the base composition of the DNA from its peak bouyant density in CsCl (28). When heat denatured DNA is centrifuged in a CsCl gradient, it moves to a density that is greater than its undenatured counterpart (29). The increase in density of denatured DNA probably relates to the fact that the single-stranded subunits form collapsed struc-

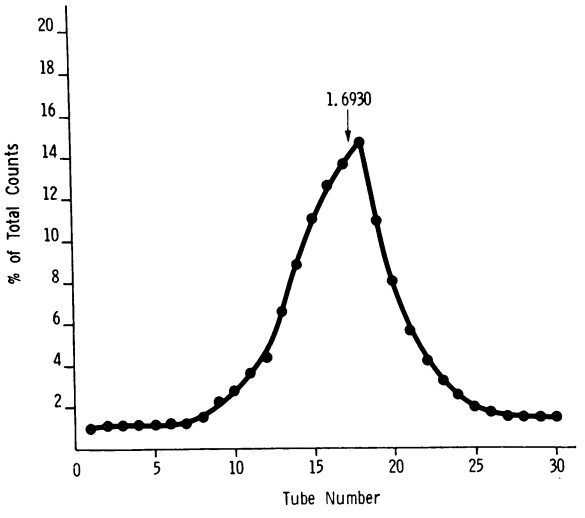

FIGURE 3 CsCl equilibrium density gradient centrifugation of unfractionated KB DNA. The density is highest on the left and lowest on the right. Peak density, 1.693 g/ml.

tures with some reformation of base pairs and therefore behave as more dense molecules because of the structural changes.

To obtain a profile of the KB DNA, 2 μg of unfractionated nucleic acid was centrifuged in a neutral CsCl gradient as shown in Fig. 3. The radioactive peak of the nucleic acid occurred at a density of 1.693 g/ml, as determined from the refractive index of the sample. This density agrees with those reported in the literature for mammalian DNA's and would correspond to a DNA with about a 61% adenine-thymine (A-T) base content (27). It is evident that the unfractionated KB DNA has a significant skew in the direction of increased density.

Salt fraction DNA was compared to alkali denatured salt fraction DNA in simultaneously centrifuged neutral CsCl gradients containing 2 μg of nucleic acid. The denatured DNA was prepared by incubating the salt fraction DNA at a concentration of 20 μg/ml of 0.25 M sodium hydroxide for 30 min at room temperature. As shown in Fig. 4, the untreated salt fraction DNA in the closed circles behaved as a nearly symmetrical band with a radioactive peak that was clearly separable from the alkali-denatured salt fraction DNA banding in a denser part of the gradient. The peak density of undenatured salt fraction DNA is identical to that of unfractionated DNA (Fig. 3). The undenatured DNA has a slight skew towards a higher density, but this is not nearly as obvious as with the unfractionated DNA.

Enzyme-treated salt fraction DNA recovered from MAK chromatography was subjected to the same centrifugation at the same concentration of nucleic acid (Fig. 5). This DNA sample centrifuged as a symmetrical band at a density about 0.005 g/ml lower than the

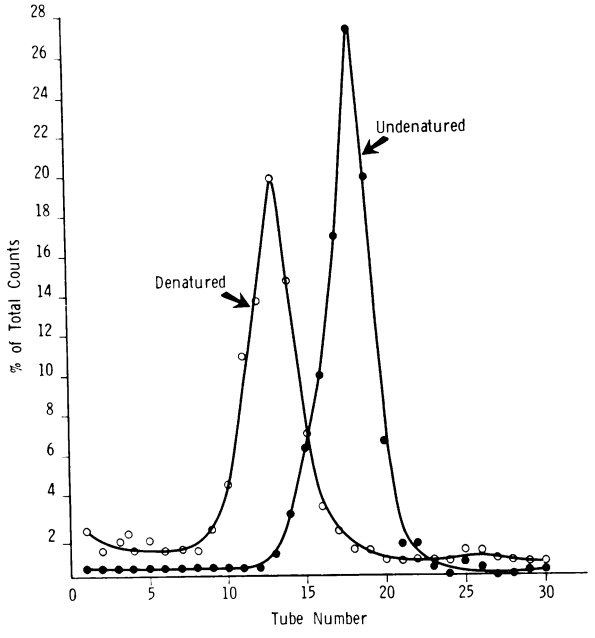

FIGURE 4 CsCl equilibrium density gradient centrifugation of salt fraction DNA. Undenatured (●——●). Alkali denatured (○——○). The density is highest on the left and lowest on the right.

nonenzyme-treated salt fraction. This density difference is within the limits of variation for the method. The salt fraction DNA does release about 1% of its radioac-

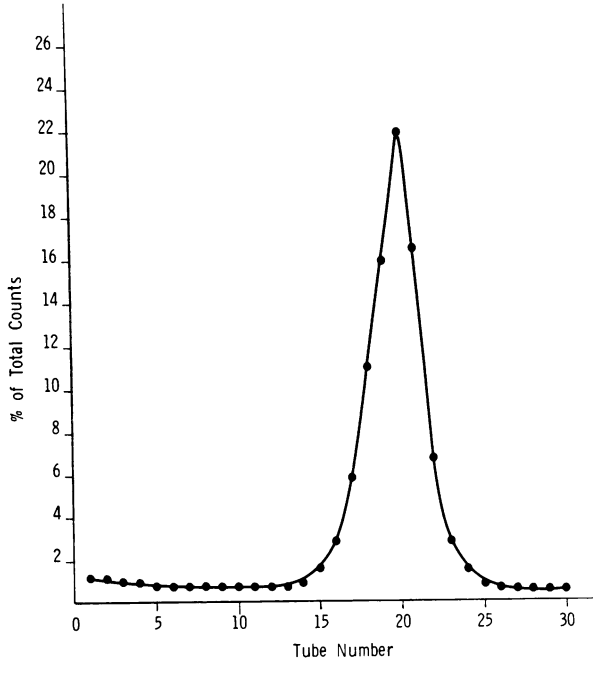

FIGURE 5 CsCl equilibrium density gradient of salt fraction DNA after endonuclease removal of single strands.

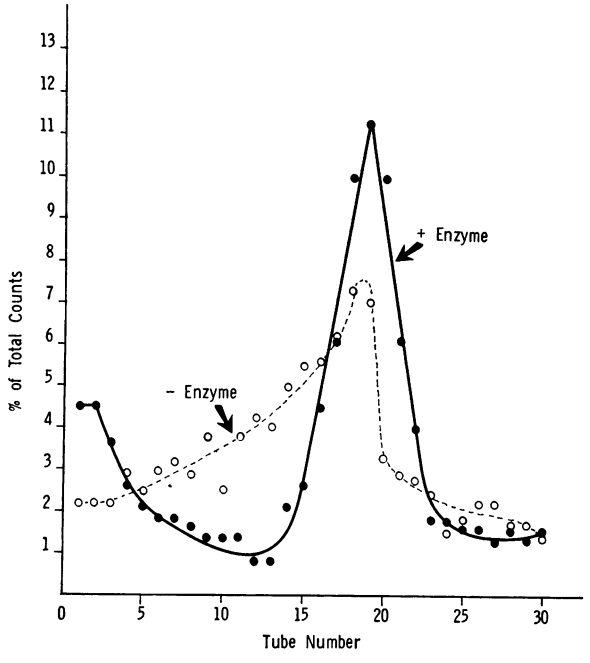

FIGURE 6 CsCl equilibrium density gradient of alkaline fraction DNA before (O- - -O) and after (●——●) endonuclease removal of single strands.

tivity with nuclease incubation, as shown in the previous section. Significantly, the salt fraction DNA does not have the obvious skew which is present in unfractionated DNA, shown in Fig. 3, indicating that the former fraction contains relatively homogeneously double-stranded nucleic acid. In the above experiments, 100% of the added radioactivity was recovered in the gradients.

For the alkaline fraction DNA, 4 μg of nucleic acid was centrifuged in a neutral CsCl gradient. As shown in the open circles in Fig. 6, this DNA fraction centrifuged with a peak density of about 0.003 g/ml less than salt fraction DNA, but there was a dramatic skew to the curve extending into the denser part of the gradient. In contrast, 3 μg of the enzyme-treated and rechromatographed alkaline fraction DNA yielded a symmetrical radioactive peak in the gradient (Fig. 6, closed circles). The endonuclease (enzyme)-treated DNA has a peak density identical with that of enzyme-treated salt fraction DNA. In the enzyme-treated sample, 100% of the added radioactivity was recovered in the gradient as opposed to 75% recovered in the nonenzyme incubated alkaline fraction. Sedimentation against the sides of centrifuge tubes results in the loss of nucleic acid with long single-stranded regions (30). This would affect the bouyant density pattern by removing nucleic acid that would add to the skew effect.

MAK profile of KB DNA before and after digestion with the UV-exonuclease. A control MAK profile of purified KB DNA was obtained by chromatographing 1 μg of nucleic acid on a 2 ml MAK column and eluting first with a 5 M salt buffer, pH 6.7, followed by a 1 M salt buffer, pH 11.6. 1-ml samples were collected and 100 μl aliquots of the fractions assayed for radioactivity. As can be seen from the profile on the left in Fig. 7, about 65% of the radioactivity eluted in the salt gradient while 35% was eluted at the alkaline pH.

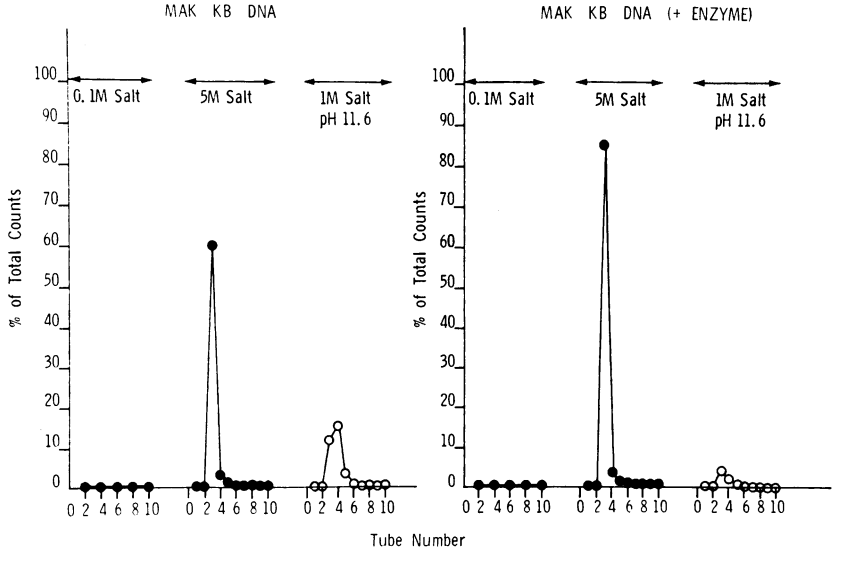

FIGURE 7 MAK profile of unfractionated KB DNA before (left panel) and after (right panel) exonuclease removal of single stranded ends.

2 μg of DNA were incubated with 10 U of UV-exonuclease in 0.6 ml of incubation mixture at 37°C for 4 h. After incubation the mixture containing the enzyme and a control mixture were sampled for total and acid soluble radioactive counts. The remaining portion (0.4 ml) of the enzyme-treated mixture was added to a 2-ml MAK column and chromatographed as described above. As can be seen from the profile on the right in Fig. 7, about 95% of the radioactivity was now eluted in the salt gradient. During incubation, the enzyme had released 3.9% of the total radioactivity contained within the nucleic acid.

The above results are virtually identical to those obtained with endonuclease digestion of single-stranded regions in KB DNA. The exonuclease released 3.9% of total radioactivity while the endonuclease, in a similar experiment, digested 2.3% of input radioactivity in the nucleic acid. After enzyme digestion of the single-stranded regions in the nucleic acid, the DNA behaved as if uniformly double-stranded on MAK chromatography as in Fig. 2. The above data indicate that the single-stranded regions of the purified KB DNA occur primarily at the ends of the molecules.

Binding of DNA to selected normal sera and sera of patients with SLE. Fig. 8 shows the dose response curve for representative sera from a normal person and a person with SLE using unseparated KB DNA. The second curve with SLE serum is the same serum sample and DNA dilution tested 3 wk later, indicating stability of the unseparated DNA. The maximum binding of DNA is approached at 0.012 μg DNA/μl of serum.

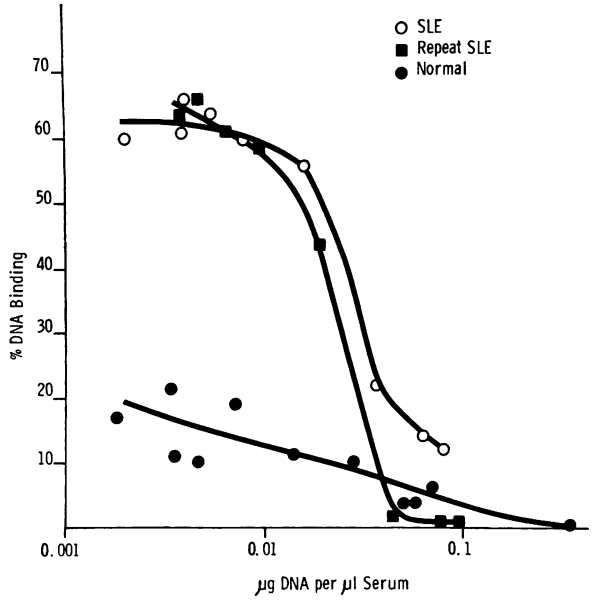

FIGURE 8 Percent DNA binding using varying amounts of DNA with normal serum (●——●) and the same SLE serum (O——O) repeated 3 wk later (■——■).

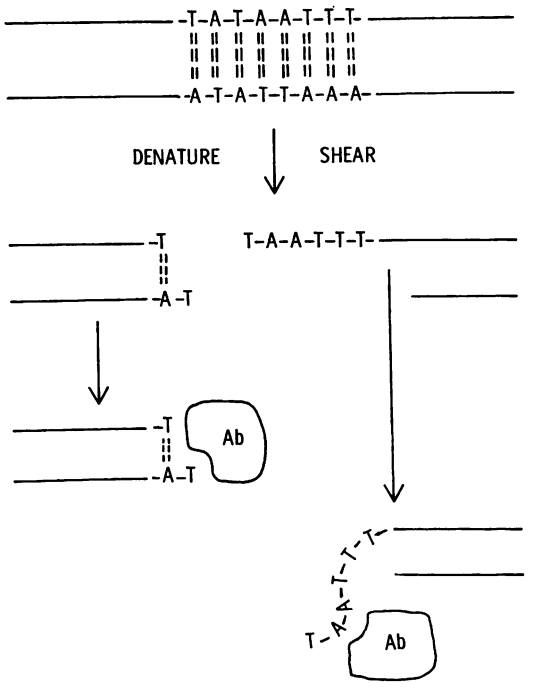

FIGURE 9 A model of DNA as antigen.

Based on dose response curves like the above, salt and alkaline fraction DNA were tested using 0.0124 and 0.0156 μg of DNA per μl of serum. Table II shows sera from patients with active SLE always bound a higher percent of salt fraction compared to alkaline fraction DNA. The reverse is true with normal sera, but the difference is small. Differences in salt and alkaline preparation and DNA concentrations probably account for the day to day variability. The percent binding of SLE sera with salt fraction DNA is different from alkaline fraction at $P < 0.01$. The difference in DNA binding between salt and alkaline fraction DNA is 50%, but as previously shown, at least 85% of the DNA in the alkaline fraction is composed of double-stranded nucleic acid. Thus, with this small sample of sera tested at different times with different MAK column preparations, the percent binding with alkaline DNA was always less than with salt DNA.

DISCUSSION

We have shown that DNA prepared from mammalian cells by the Marmur technique is a pure preparation of nucleic acid that is composed primarily of two populations of molecules. One of the populations consists of primarily double-stranded nucleic acid while the other population is of double-stranded nucleic acid with significant single-stranded regions. Techniques such as the Marmur procedure designed to yield purified preparations of DNA invariably cause shear breakage of the

TABLE II

Binding of Normal Sera and Sera from Patients with SLE with Salt and Alkaline Fraction KB DNA

Serum tested	Date tested	DNA binding	
		Salt DNA	Alk DNA
		%	
SLE			
Gu	2/9	71	16
	2/26	64	24
	3/7	75	22
	3/5	68	25
Ge	2/9	55	24
	2/26	67	50
Wa	2/15	67	37
	3/7	70	45
Ne	2/15	66	33
St	2/15	69	47
Ma	3/5	57	39
Mean		66	33
Normal			
I	2/9	3	11
	2/26	1	12
	3/5	16	27
	3/7	27	28
P	2/15	14	15
	3/5	16	21
S	2/15	5	11
	2/26	13	22
	3/5	15	24
Ak	2/15	6	9
	3/5	11	20
M	2/9	15	18
Ga	2/15	8	16
Mean		12	18

* All fractions bound with 0.0124 to 0.158 μg DNA per μl serum.

nucleic acid during the isolation procedure (31). This shear breakage causes fragmentation of the high molecular weight intracellular DNA in probably the same manner that high molecular weight purified DNA can be broken into smaller fragments by a variety of physical techniques (32, 33). Investigators have been aware of this heterogeneity of purified mammalian DNA and have used MAK chromatography to separate double-stranded molecules from denatured or single-stranded molecules contained within the preparations. In the literature the terms denatured DNA and single-stranded DNA have been used interchangeably to describe nucleic acid preparations used in anti-DNA tests (2, 6). In the investigation of antibodies to DNA found in the sera of patients with SLE, investigators have described serum preparations that bind to double-stranded DNA and/or denatured DNA (1, 2, 9). In contrast to the latter finding, experimentally produced antibodies react preferentially with denatured nucleic acid (8). We believe that a major part of the issue of serum antibody binding to DNA revolves around the adequacy of the terms "single-stranded," "denatured," and "double-stranded" in describing nucleic acid preparations including those fractionated by the various chromatographic and filtration techniques. Our approach to the problem of serum antibody binding to DNA is presently directed primarily at the nucleic acid antigen which we feel has been inadequately characterized.

Our results show that the purified fraction of mammalian DNA that behaves as if it were "uniformly denatured nucleic acid" on MAK columns, that is, elutable only when the pH is raised to an alkaline value, does in fact contain at least 85% double-stranded regions, as determined by endonucleolytic enzyme specific digestion of single-stranded DNA. Endonuclease or exonuclease removal of the single-stranded regions results in the remaining DNA behaving as double-stranded DNA on MAK chromatography as indicated by elution in the salt gradient. The alkaline elutable nucleic acid behaves as if mostly denatured in CsCl bouyant density centrifugation, but after enzyme removal of the single strands, the alkaline fraction DNA has a bouyant density and band profile identical to that of double-stranded DNA. It is obvious then that double-stranded DNA with single-stranded areas can behave as if it were denatured nucleic acid on MAK chromatography elution and CsCl equilibrium density centrifugation.

The chromatographically purified double-stranded DNA (nucleic acid that is salt elutable from a MAK column) does contain small single-stranded regions as measured by incubation of the nucleic acid with the single-strand specific enzyme. This structural defect of apparently uniformly double-stranded nucleic acid accounts for the fact that purified native DNA elutes over a broad neutral salt range on MAK chromatography, and that enzyme removal of single strands from the salt elutable nucleic acid results in a significantly narrowed profile on repeat MAK chromatography. Our MAK chromatographic profiles of salt and alkaline-elutable DNA reveal that both fractions are heterogeneous, and that this heterogeneity is probably solely due to the presence of single-stranded regions of variable length.

In addition, we have found that initial fractionation of relatively large quantities of DNA on MAK columns

may be incomplete in that it does not strictly separate double-stranded DNA from double-stranded DNA with significant single-stranded regions. We cannot explain this observation except that it may relate to the extent that double-stranded and single-stranded regions are able to bind to the methylated albumin on any given passage through the chromatographic column. In addition, concentrated DNA suspensions have a tendency to form aggregates which could influence the binding of double-stranded and single-stranded regions to the column (34). The double-stranded DNA with single-stranded regions, alkaline fraction DNA, may behave as if denatured in CsCl density centrifugation (Fig. 6) suggesting that the molecule undergoes conformational changes which could lead to variable binding to the chromatographic suspensions depending upon the configuration of the nucleic acid. Endonuclease treated salt (Fig. 5) and alkaline (Fig. 6) fraction DNA have the same peak bouyant densities. This indicates that the double-stranded regions in the two fractions have the same average base content. This suggests that base content alone does not account for the differences in binding to the column.

Of particular interest is the fact that the alkaline fraction DNA, while releasing over 70% of its radioactivity to digestion by the single-strand specific enzyme, loses only about 10% of its total nucleic acid to enzyme incubation. The salt fraction DNA releases 1% of its radioactive counts and a nonmeasurable amount of nucleic acid to enzyme digestion of single-stranded regions. Since our DNA was labeled with tritiated thymidine, we have concluded from these findings that the single-stranded regions have a high thymine or adenine and thymine content. We doubt that nonrandom labeling is a cause of the above results because (*a*) the growing cells had gone through two doublings before harvesting, and (*b*) the base content of the double-stranded regions of both fractions is identical. However, it is possible that thymine-rich regions may be preferentially labeled because they serve as growing points during DNA replication. It has been shown in nucleic acid denaturation studies that A-T base pairs separate more easily than guanine-cytosine (G-C) base pairs, presumably on the basis that the A-T base pairs have two hydrogen bonds while the G-C base pairs have three hydrogen bonds between the bases (35). We suggest that the regions of the DNA that are relatively rich in A-T base pairs are more susceptible to denaturation and shear during isolation, and that these regions form the preferential point of breakage of the nucleic acid during purification.

To identify the location of the single-stranded regions in the nucleic acid, we have employed the enzyme UV-exonuclease which will digest any single-stranded ends of DNA. The action of UV-exonuclease on the unfractionated mammalian nucleic acid was similar to that of the endonuclease in terms of percent radioactivity made acid soluble and MAK profile of the DNA before and after enzyme treatment. We propose that the single-stranded regions occur primarily at the ends of the nucleic acid but some may be present in the interior of the molecules. This suggestion is consistent with the finding of single-stranded ends on fragments of high molecular weight DNA sheared by mechanical techniques (32, 33).

The chromatographic separation and changes after enzyme digestion of DNA are not peculiar to mammalian DNA since we have repeated most of the experiments described in this paper with *E. coli* DNA and obtained the same results.

In our experiments on the binding of the chromatographically purified fractions of mammalian DNA to the sera of patients with SLE, we have found that the sera bind significantly less to the alkaline fraction DNA than the salt fraction DNA. This difference in binding clearly does not represent simply an affinity for double-stranded vs. single-stranded DNA since the alkaline fraction DNA is at least 85% double-stranded nucleic acid. Our data would appear also to exclude overall base content of the nucleic acid as a primary factor in the relationship of antibody binding to DNA, consistent with the results reported by Koffler, Carr, Agnello, Thoburn, and Kunkel (1). Our conclusions are admittedly based on a small number of sera which were selected for high DNA antibody content by the hemagglutination test (3). However, using hydroxyapatite purified DNA fractions, Tan and Epstein showed that 17 of 23 SLE sera bound less with the "denatured" and more with the "native" fraction (5).

Many investigators have shown that the sera of patients with SLE may have variable activity in that some sera will react with (*a*) native and denatured DNA, (*b*) with native DNA only, and (*c*) with denatured DNA only (1, 2, 9). Tan and Natali purified calf thymus DNA by a single passage through a MAK column and showed that SLE sera bound preferentially to the salt-elutable calf thymus DNA and not to the alkaline-elutable fraction, suggesting that the antibody in their SLE sera was indeed specific for double-stranded DNA (8). Our experiments suggest that even chromatographically fractionated DNA preparations such as reported in the literature cannot be equated simply with single-stranded or double-stranded nucleic acid. It seems apparent that the variable binding activity reported with various SLE sera may not reflect simply specificity towards double-stranded vs. single-stranded DNA, but rather points to differential binding of antibody to specific regions of structurally heterogeneous DNA.

Specifically, we think that this variable binding is a reflection of variable length single-stranded regions occurring in DNA that is primarily double-stranded nucleic acid. We have shown that salt and alkaline fraction DNA have the same average molecular weight by sucrose gradient centrifugation, which would rule out size as an important factor in the binding differences (36). We have not ruled out a conformational change in the double-stranded regions due to the single-stranded ends in alkaline fraction DNA as a cause for the binding differences.

As mentioned, our data suggest that these single-stranded regions are rich in thymine. This probable base content of the single-stranded regions is important as suggested by the following experiments in the literature. Koffler and coworkers have shown that sera with antibody to double-stranded DNA are inhibited by the synthetic single-strand polymer poly dA, less so by poly dT, and least by the polymer poly dC (1). Stoller, Levine, Lehrer, and Van Vunakis have shown that tetranucleotides of thymine inhibit complement fixation by antibody to denatured DNA, and that this inhibition falls off with tetranucleotides enriched with cytosine rather than thymine (37). Additionally, these authors showed that the serum antibody did not fix complement in the presence of denatured synthetic poly dAT. These data point to the important influence of single-stranded areas rich in thymine or thymine and adenine in the reaction of some SLE antibodies to nucleic acid. The antibodies in patients with SLE may be directed to a large degree at the ends of "native" DNA that contain short single-stranded regions rich in thymine and also at areas of bifurcation between double-stranded DNA and A-T rich single-stranded regions in denatured DNA. Therefore, we propose that the antigenic site in DNA, to which antibodies against native DNA are directed, contains both double-stranded and single-stranded nucleic acid. This suggestion would explain why synthetic polymers rich in adenine or thymine or denatured DNA with its single-stranded regions relatively rich in A-T might inhibit the SLE antibodies to primarily double-stranded DNA but not necessarily cross-react with it. This inhibition could take place by the base pairing of the polymers or single-stranded regions in denatured DNA to the single-stranded ends of the double-stranded DNA, thereby covering up the antibody binding site; or the polymers could inhibit the antibody binding to double-stranded DNA by reacting with only a portion of the antibody combining site.

The simplest explanation for our results and those in the literature is outlined in the model represented in Fig. 9. The DNA molecule shears at A-T rich regions. At least two populations of molecules are produced: (*a*) one with very short ends that behaves as double-stranded DNA and (*b*) another with long ends that behaves like denatured DNA. This model suggests that antibodies in patients with SLE to double-stranded DNA may react at the ends of the molecules in the region where the molecule is rigid, but where the nucleotides are in part exposed. Note that short single-stranded regions may be part of the binding site. However, alkaline-elutable DNA has long single-stranded ends which will curve around the end of the DNA and prevent the interaction of antibodies with the molecule. Several predictions of this model are being tested. Polynucleotides of thymine and/or adenine might inhibit the binding of SLE sera to salt DNA. Truly circular DNA, such as SV-40 DNA, should react very little with anti-DNA antibody. Also, enzyme digestion of alkaline fraction DNA should lead to a marked increase in percent binding with SLE sera containing anti-DNA antibody reactive with salt DNA. Sera reactive with only single-stranded DNA should not bind well with salt fraction DNA, and binding with alkaline fraction DNA should be markedly decreased by enzyme digestion of the single-stranded regions from alkaline fraction DNA.

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