A Human Centromere Antigen (CENP-B) Interacts with a Short Specific Sequence in Alphoid DNA, a Human Centromeric Satellite

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Abstract. We report the interaction between a human centromere antigen and an alphoid DNA, a human centromeric satellite DNA, which consists of 170-bp repeating units. A cloned alphoid DNA fragment incubated with a HeLa cell nuclear extract is selectively immunoprecipitated by the anticentromere sera from scleroderma patients. Immunoprecipitation of the DNA made by primer extension defines the 17-bp segment on the alphoid DNA that is required for formation of DNA-antigen complex. On the other hand, when proteins bound to the biotinylated alphoid DNA carrying the 17-bp motif are recovered by streptavidin agarose and immunoblotted, the 80-kD centromere antigen (CENP-B) is detected. DNA binding experiments for proteins immunoprecipitated with anticentromere serum, separated by gel electrophoresis, and transferred to a membrane strongly suggest that the 80-kD antigen specifically binds to the DNA fragment with the 17-bp motif. The 17-bp motif is termed the "CENP-B box." Alphoid monomers with the CENP-B box are found in all the known alphoid subclasses, with varying frequencies, except the one derived from the Y chromosome so far cloned. These results imply that the interaction of the 80-kD centromere antigen with the CENP-B box in the alphoid repeats may play some crucial role in the formation of specified structure and/or function of human centromere.

THE centromere plays an essential role in mitotic and meiotic segregation. The centromeric region of mitotic chromosomes of higher eukaryotes is structurally specified by the primary constriction at which sister chromatids associate and a pair of kinetochores, the anchorage sites of the spindle microtubules, are formed (Rieder, 1982). Ultrastructural studies revealed that the kinetochore consists of a trilaminar disk (Jokelainen, 1967; Comings and Okada, 1971; Roos, 1973) that is composed, at least in part, of highly organized chromatin fibers to which microtubules attach directly (Ris and Witt, 1981; Rattner, 1987). It has been shown that the centromere-kinetochore region of mammalian chromosomes is recognized by sera from certain scleroderma patients (Moroi et al., 1980). Centromere antigens are detected by antibodies in the sera as doublets at centromere-kinetochore regions in metaphase chromosomes or as prekinetochore speckles in interphase nuclei (Moroi et al., 1981; Masumoto et al., 1989). By immunoelectron microscopy, centromere antigens were located at the trilaminar kinetochore structure of mitotic PtK2 chromosomes (Brenner et al., 1981). Three centromere antigens, namely, 17- (CENP-A),[1] 80- (CENP-B), and 140-kD (CENP-C) proteins, are most commonly detected in human chromosomal proteins by immunoblotting analyses (Earnshaw et al., 1985; Valdivia and Brinkley, 1985). Palmer et al. (1987) suggested that the 17-kD antigen, which was copurified with nucleosome core particles and with histones, may be a histone-like component of a centromere-specific nucleosome. For the 80-kD antigen, cDNA clones composing >90% of the coding sequence have been isolated (Earnshaw et al., 1987), although the function of the gene has not been clarified.

With regard to *cis*-acting DNA sequences specifying centromere structure and/or function, a functional centromere sequence has been identified in *Saccharomyces cerevisiae* (Clarke and Carbon, 1980, 1985). A 120-bp-long centromere sequence is composed of three consensus elements (Hieter et al., 1985), of which element III (a conserved 25-bp sequence) is essential for mitotic segregation and protein binding (McGrew et al., 1986; Ng and Carbon, 1987; Saunders et al., 1988). In *Schizosaccharomyces pombe*, minichromosomes containing a 65- or 150-kb centromeric DNA segment were constructed (Hahnenberger et al., 1989). In contrast, no specific DNA sequences have been correlated with function of the centromere in other eukaryotic organisms. In mammalian cells, satellite DNAs were localized by in situ hybridization predominantly to the centromeric heterochromatin or to the heterochromatic arms of chromosomes (Pardue and Gall, 1970; Jones and Corneo, 1971; Beridze, 1986; Wong and Rattner, 1988). Alpha satellite DNA, or alphoid DNA, is a long, tandemly repeated DNA family based on a 170-bp fundamental monomer repeat unit (Manuelidis, 1976, 1978; Wu and Manuelidis, 1980). The alphoid DNA family in human is composed of many subclasses, which share varying degrees of sequence homology (60–99%) and have been local-

1. *Abbreviations used in this paper*: CENP, centromere antigen; polyvinyldene difluoride.

ized to the primary constriction (centromere) of specific chromosomes (Willard and Waye, 1987; Alexandrov et al., 1988).

In a previous work (Masumoto et al., 1989), we constructed a human genomic DNA library from the fraction of chromosomal segments associated with centromere antigens. From this we isolated an alphoid DNA clone (clone 4-1 containing an alphoid dimer, α341) that hybridized specifically to the centromere regions of about one-third of human chromosomes. In situ hybridization and indirect immunofluorescent staining executed on the same preparation showed that the sites of the alphoid DNA overlapped perfectly with the sites of centromere antigens throughout the cell cycle, even in artificially extended metaphase chromosomes. These results suggested that alphoid DNA clusters may interact with centromere antigens at multiple sites.

In this paper, we demonstrate that a specific alphoid DNA segment indeed interacts with a centromere antigen in a HeLa cell nuclear extract using immunoprecipitation with the anticentromere sera. A 17-bp segment of the alphoid DNA is required for immunoprecipitation. The results suggest that the 80-kD centromere antigen (CENP-B) binds to the DNA fragment carrying the 17-bp motif (CENP-B box).

Materials and Methods

Cell Culture

HeLa cells were grown in DME medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% calf serum (Flow Laboratories, North Ryde, Australia) at 37°C under 5% CO_2.

Antisera

Anticentromere (kinetochore) sera from scleroderma patients A. K. and Y. were provided by Yasuoki Moroi (Tokyo University, Tokyo, Japan) and those from patients S. T., H. M., R. K., T. M., K. G., I. H., and H. K. by Masaru Ohashi (Nagoya University, Nagoya, Japan). By immunoblotting of the proteins of isolated HeLa nuclei and chromosomes, all these patients' sera recognize three centromere antigens (17, 80, and 140 kD) in common, but the titer for the 80- or the 140-kD antigen is lower in sera T. M. or I. H., respectively, than in others and the titers for the 17-kD antigen of the sera H. M. and R. K. are very low. Normal human sera (K, S, N) that do not recognize any centromere antigens were obtained from Y. Moroi.

Phage and Plasmid DNAs

A derivative of λgt10 carrying a 341-bp-long alphoid DNA segment (α341 DNA) was described previously (Masumoto et al., 1989). The 341-bp Bam HI fragment was recloned into the Bam HI site of pUC119 (Vieira and Messing, 1987) and the resulting plasmid, pUCα(4–1) or pUCα$(4–1)_2$, carries a single or two tandem copies of α341 DNA. To construct plasmids pUCα169, pUCα120, pUCα49, and pUCα172, plasmid pUCα$(4–1)_2$ was cleaved by Dde I and the alphoid subfragments, whose ends were filled in with *Escherichia coli* DNA polymerase I Klenow fragment (Takara Shuzo, Kyoto, Japan), were ligated with the Sma I-cleaved pUC119. M13 α169R and M13 α169L were constructed by inserting the 169-bp Dde I fragment from plasmid pUCα(4–1), whose ends were filled in with DNA polymerase I Knenow fragment, into the Sma I site of M13mp19 RF DNA in both orientations. Plasmid L1.26 carries an 849-bp Eco RI fragment of alphoid DNA derived from chromosome 13 or 21 (Devilee et al., 1986). Plasmid pXBR-1, which has a 2-kb alphoid Bam HI fragment from the X-chromosome was provided by B. Hamkalo (Yang et al., 1982). Plasmid and phage DNAs were prepared as described (Maniatis et al., 1982).

Preparation of HeLa Nuclei

All steps were done below 4°C except as indicated. HeLa cells were released from confluent monolayer by trypsin treatment (0.2%) and collected by centrifugation at 1,000 rpm for 5 min. The cells were then washed once with DME plus 10% calf serum, twice with PBS and finally twice with isolation buffer (3.75 mM Tris/HCl, pH 8.0, 0.05 mM spermine, 0.125 mM spermidine, 0.5 mM EDTA, 0.5 mM DTT, 20 mM KCl, 0.1 mM PMSF [Sigma Chemical Co., St. Louis, MO]). The cell pellet was resuspended with 5 vol of isolation buffer supplemented with 0.1% digitonin (WAKO Pure Chemical, Osaka, Japan). The cell membrane was broken using a Dounce homogenizer for 15 strokes and the lysate was centrifuged for 10 min at 1,500 rpm. The nuclear pellet was resuspended and washed twice with isolation buffer supplemented with 0.1% digitonin and then once with washing buffer (20 mM Hepes, pH 8.0, 20 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 0.1 mM PMSF). Nuclei were collected by centrifugation for 5 min at 1,200 rpm, and immediately used for protein extraction.

Preparation of Extracts from HeLa Nuclei

The isolated HeLa nuclei were resuspended with washing buffer (~2.7×10^8 nuclei/ml) and mixed with 1 vol of extraction buffer (20 mM Hepes, pH 8.0, 0.5 mM EDTA, 0.5 mM DTT, 0.1 mM PMSF, 0.5 μg/ml pepstatin A [Boehringer Mannheim Biochemicals, Indianapolis, IN]) supplemented with NaCl and glycerol at final concentrations of 0.5 M and 15% (vol/vol), respectively. After 1 h at 0°C with gentle agitation, the suspension was centrifuged at 25,000 rpm for 1 h in a rotor (Type 40; Beckman Instruments, Fullerton, CA). The pellet was resuspended and extracted again with extraction buffer containing 2 M NaCl (0.5–2 M NaCl extract) by the same procedure. The supernatants at each centrifugation step and the final pellet were examined by immunoblotting with anticentromere serum. The supernatants were assayed for DNA binding activity either immediately or after storage at −70°C. The 0.5 M NaCl and the 0.5–2 M NaCl extracts contained 6.2 and 2.5 mg proteins per ml, respectively. In some experiments, the fraction extracted with the buffer containing 0.25 M NaCl (0.25 M NaCl extract) or that extracted with 0.5 M NaCl after 0.25 M NaCl extraction (0.25–0.5 M extract) was used.

SDS-PAGE and Immunoblotting

After SDS-PAGE (Laemmli, 1970), proteins were transferred to an polyvinyldene difluoride (PVDF) membrane (Millipore Corp., Bedford, MA) according to Towbin et al. (1979), omitting SDS. The membrane was preincubated with T-PBS (154 mM NaCl, 10 mM Na-phosphate buffer, pH 7.6, 0.05% Tween-20) containing 3% nonfat dry milk (Gibco Laboratories, Grand Island, NY) for 2 h at room temperature, and then incubated at 4°C overnight with antibody solution (1% gelatin, 20 mM Tris/HCl, pH 7.6, 500 mM NaCl) containing an anticentromere serum (at 1:1,000 dilution). The membrane was washed five times for 20 min each with T-PBS at room temperature and incubated at room temperature for 2 h with biotinylated rabbit anti-human IgG (Vector Laboratories, Burlingame, CA) at 1:1,000 dilution with antibody solution. After being washed five times for 20 min each with T-PBS, the membrane was incubated for 1 h at room temperature with avidin (DH grade) and biotinylated horseradish peroxidase complex (Vector) according to the manufacturer's instructions. The membrane was washed again and color development with chloronaphtol and H_2O_2 was carried out.

DNA Binding Assay by Gel Retardation

Plasmids carrying alphoid sequence were digested with appropriate restriction endonucleases, and the fragments were end-labeled with DNA polymerase I Klenow fragment, [α-^{32}P]dCTP or dTTP (3,000 Ci/mmol; Amersham International, Amersham, UK) and the other three dNTPs. 0.5 ng of end-labeled DNA fragments was incubated with excess (0.5 or 2 μg) poly(dI·dC)·poly(dI·dC) and various amounts of 0.5 M nuclear extract in 20 μl of binding buffer (10 mM Tris/HCl, pH 8.0, 10% [vol/vol] glycerol, 1 mM EDTA, 1 mM DTT, and 150 mM NaCl at final concentrations) for 1–1.5 h at 24°C. Incubation with 0.5–2 M NaCl extract was carried out while dialyzing the reaction mixture against binding buffer. Then the mixtures were electrophoresed on agarose gels (1.5% agarose, 40 mM Trizma base, 12.5 mM NaOAc, 3.3 mM EDTA, adjusted to pH 7.9 with HCl) at 3 V/cm, 4°C according to Berman et al. (1986), and gels were dried and exposed to Kodak XAR-5 film.

DNA Immunoprecipitation Assay

The DNA-protein complexes formed under the appropriate conditions determined by the gel retardation assay were examined by immunoprecipitation assay using anticentromere serum as originally described by Mckay (1981). Briefly, end-labeled DNA fragments (5 ng) were incubated with 0.5 M NaCl extract (19 μg proteins) or 0.5–2 M NaCl extract (7.5 μg proteins) in 100 μl of binding buffer supplemented with 50 μg/ml poly(dI·dC)·poly-

(dI·dC) and 0.05% (vol/vol) NP-40 for 1 h at 24°C. Then 1 μl of anticentromere serum was added and the mixture was incubated for 30 min on ice to form immune complexes. 25 μl of protein A-Sepharose (0.2 g/ml) (Pharmacia Fine Chemicals, Uppsala, Sweden) was added and the mixture was incubated for 30 min on ice with gentle agitation. After being washed three times with 0.5 ml of binding buffer containing 0.5% (vol/vol) NP-40, the pellet was suspended in a solution containing 1% SDS, 50 mM NaCl, 10 mM Tris/HCl (pH 8.0) and 1 mM EDTA. The DNA was purified by extraction with phenol and with $CHCl_3$, followed by ethanol precipitation, and was electrophoresed in an agarose gel. The gel was subjected to autoradiography after drying.

Primer Extension DNA Immunoprecipitation

The procedures described by Hawley-Nelson et al. (1988) were used with some modifications. Dideoxy chain termination reactions were carried out on single strand M13α169R or M13α169L DNA using modified T7 DNA polymerase (Sequenase kit; United States Biochemical Corp., Cleveland, OH) and [α-^{32}P]dCTP as described by the manufacturer. After primer extension, 2.5 μl of reaction mixture containing 0.1 μg of template DNA was added to 50 μl of 150 mM sodium acetate (pH 4.7), 900 mM NaCl, 30 mM zinc acetate and 50 units of mung bean nuclease (Takara Shuzo, Kyoto, Japan) and incubated at 22°C for 30 min. After addition of 50 μl 200 mM Tris/HCl (pH 8.0), 60 mM EDTA, and 100 μg/ml yeast tRNA to stop the reaction, DNA was precipitated with ethanol and resuspended in 10 μl of 10 mM Tris/HCl (pH 7.4), 1 mM EDTA and 10 mM NaCl. Half of each sample was used for DNA immunoprecipitation using the 0.5 M NaCl nuclear extract as described above. The precipitated DNA was resuspended in 8 μl loading buffer (United States Biochemical Corp.), heated to 95°C for 3 min and analyzed by electrophoresis on a 6 or 8% polyacrylamide gel containing 8 M urea.

Detection of a Centromere Antigen Bound to Biotinylated DNA

Three kinds of DNA fragments generated by Dde I digestion, α169 from pUCα(4–1), α172 from pUCα(4–1)$_2$, and a 166-bp fragment from pUC119, were purified by agarose gel. The ends of the fragments were filled in with Klenow fragment, biotin-11-dUTP (Enzo Corp., NY) dCTP, dGTP, dATP, and [α-^{32}P] dCTP, and the DNA fragments were purified by Sephadex G-50 and precipitated with ethanol in the presence of 10 μg of poly-(dI·dC)·poly(dI·dC). The biotinylated DNA fragments (0.25 μg, 2 pmol) were incubated with 0.25–0.5 M NaCl nuclear extract (4.4 mg proteins, 2 × 10^8 nuclei) in binding buffer (5 ml) supplemented with 200 μg/ml poly-(dI·dC)·poly(dI·dC), 0.05% NP-40 for 1 h at 24°C.

The biotinylated DNA-protein complex was absorbed with streptavidin-agarose beads as described by Chodosh et al. (1986). In short, streptavidin agarose beads (100 μl packed vol, Bethesda Research Laboratories, Gaithersburg, MD) were incubated with 3 vol of binding buffer with 0.25 mg/ml BSA (Sigma Chemical Co.) and 200 μg/ml poly(dI·dC)·poly(dI·dC) for 30 min at 4°C, washed with binding buffer and used immediately. After addition of streptavidin-agarose, the binding mixtures were incubated for 4 h at 4°C with gentle rotation. The agarose beads were then pelleted by brief centrifugation, washed five times with Binding Buffer supplemented with 0.5% NP-40, and packed into a column. The proteins were eluted from the column with 0.5 ml elution buffer (10 mM Tris/HCl, pH 8.0, 20% glycerol, 1 mM EDTA, 1 mM DTT, 0.05% NP-40) supplemented with 0.5 M NaCl, and 50-μl fractions were collected. Fractions were assayed by DNA immunoprecipitation as described above and stored at −70°C.

DNA Binding Activity of Proteins Immobilized to PVDF Membrane

Both strands of 56-bp DNA (nucleotide positions 231–286 in Fig. 6) chemically synthesized were annealed, end-labeled by T4 polynucleotide kinase (Takara Shuzo) with [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham International) and used as a probe (1.4 × 10^6 cpm/pmol) for the DNA binding assay. Nuclear proteins were partially purified with the anticentromere antibody conjugated to Sepharose 4B as follows. IgG fraction purified from a patient serum I. H. with Q-Sepharose (Pharmacia Fine Chemicals) was conjugated with CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) (10 mg IgG/1 ml gel) (Johnstone and Thorpe, 1982). The 0.5 M NaCl HeLa nuclear extract (1.8 ml, 1.8 × 10^8 nuclei) were mixed with 40 μl of the antibody-conjugated Sepharose and incubated for 12 h at 4°C with gentle agitation. Sepharose beads were pelleted and washed three times with NTE (10 mM Tris·HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 0.1% NP-40) supplemented with 0.5 M NACl and once with NTE containing 0.1 M NaCl. The proteins bound to the antibody-conjugated Sepharose were dissolved in sample buffer and electrophoresed without boiling on SDS polyacrylamide gel (Laemmli, 1970). Proteins in the gel were transferred to PVDF membrane (Towbin et al., 1979) using the buffer without SDS and methanol. The membrane was blocked with 5% nonfat dry milk in 20 mM Tris/HCl (pH 8.0) for 1 h at 24°C. The proteins bound to the membrane were denatured with 7 M guanidine/HCl in 50 mM Tris/HCl (pH 8.0), 50 mM DTT, 2 mM EDTA, 0.25% nonfat dry milk for 1 h at 24°C, and then renatured in 50 mM Tris/HCl (pH 8.0), 1 mM DTT, 1 mM EDTA, 100 mM NaCl, 0.25% nonfat dry milk for 16 h at 4°C according to the procedure of Celenza and Carlson (1986). The membrane blocked again as described above was incubated with the end-labeled 56-bp DNA (10^6 cpm/27 ng) in 1 ml of binding buffer II (10 mM Tris/HCl, pH 8.0, 1 mM DTT, 1 mM EDTA, 0.05% NP-40, 0.25% nonfat dry milk) supplemented with 50 mM NaCl for 2 h at 24°C with gentle agitation. Where specified, competitor DNA was added to the binding reaction. After being washed five times with binding buffer II containing 100 mM NaCl for 1 h at 24°C, the membrane was subjected to autoradiography.

Results

Centromere Antigens in Crude Nuclear Extracts

We first determined conditions for the extraction of centromere-specific antigens from HeLa nuclei. Whole nuclei or nuclear extracts prepared at various salt concentrations were fractionated by PAGE and antigens were detected by immunoblotting (Fig. 1). Three major antigens, 17- (CENP-A), 80- (CENP-B), and 140-kD (CENP-C), were detected in the whole nuclear extract (Fig. 1 *B*, lane *1*). The majority of 80 and 140 kD, but not 17-kD antigens, was extracted with 0.5 M NaCl buffer (Fig. 1 *B*, lane *2*). In contrast, the subsequent extraction with 2 M NaCl (referred to as 0.5–2 M NaCl extract) recovered approximately half of the 17-kD antigen (Fig. 1 *B*, lane *3*) and the rest of the antigen remained in the nuclear pellet (Fig. 1 *B*, lane *4*). None of these major centromere antigens were extracted with 0.25 M NaCl from isolated nuclei (Fig. 1 *B*, lane *5*). In the following experiments to examine the interaction of proteins with alphoid DNA, we used 0.5 M extract that contains 80- and 140-kD antigens or 0.5–2 M extract containing the 17-kD antigen.

Binding of Nuclear Protein(s) to Alphoid DNA

To examine the specific binding of nuclear proteins to alphoid DNA, we used a derivative of pUC119, pUCα (4-1), which carries a 341-bp-long alphoid dimer sequence at the Bam HI site (Fig. 2). The plasmid DNA was digested with restriction enzymes Bam HI and Apa LI, and end-labeled with [α-^{32}P]-dCTP as described in Materials and Methods. The labeled DNA fragments, alphoid dimers (α341), and four fragments derived from pUC119 DNA, were incubated with various amounts of the 0.5 M or 0.5–2 M NaCl extract in the presence of excess amounts of poly(dI·dC)·poly(dI·dC). Formation of nuclear protein-DNA complexes was examined by retardation of the mobility of radioactive DNA fragments in an agarose gel electrophoresis (Fig. 3). Upon incubation with a certain amount of 0.5 M NaCl extract, the amount of α341 fragment electrophoresing at its original mobility greatly decreased, whereas movements of the other DNA fragments were little affected (Fig. 3 *A*, lane *3*). Under the conditions used, the shifted α341 DNA gave a smear which is not clearly seen in Fig. 3 *A* (data not shown). In contrast, when the 0.5–2 M NaCl extract was used, no specific shift of the alphoid

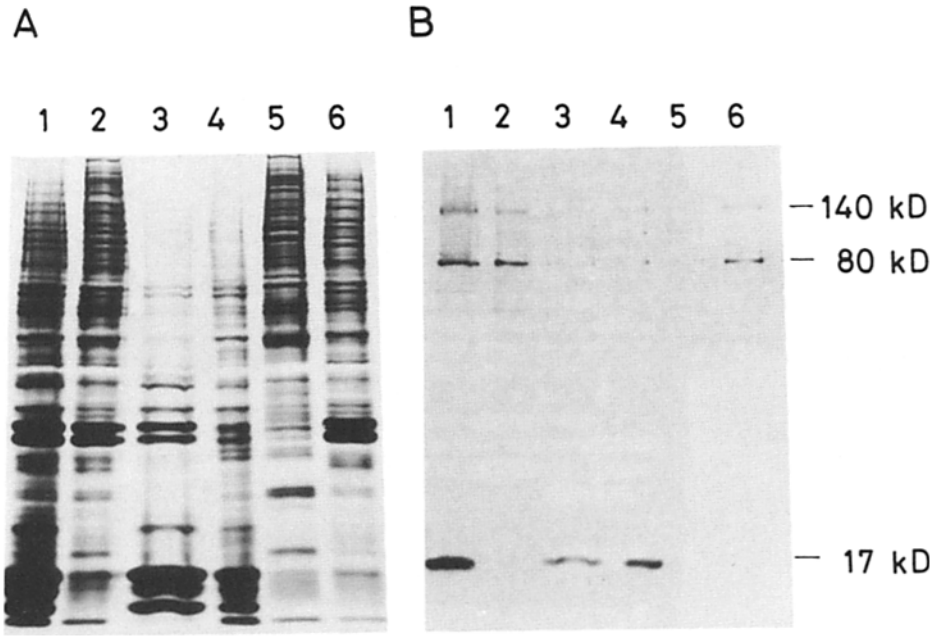

Figure 1. Analysis of centromere antigens in extracts from HeLa nuclei. Proteins in whole nuclei and nuclear extracts (4×10^5 nuclei) were electrophoresed in duplicate on 10% polyacrylamide gels containing SDS. (*A*) Silver staining of proteins. (*B*) Immunoblotting showing the distribution of centromere antigens. The anticentromere serum from patient S.T. was used. Lane *1*, whole nuclei; lane *2*, nuclear extract with 0.5 M NaCl; lane *3*, reextract with 2 M NaCl after extraction with 0.5 M NaCl (0.5–2 M NaCl extract); lane *4*, the pellet after 2 M NaCl extraction; lane *5*, nuclear extract with 0.25 M NaCl; lane *6*, reextract with 0.5 M NaCl after extraction with 0.25 M NaCl (0.25–0.5 M NaCl extract).

fragment was observed with any amount of the extract tested (Fig. 3 *B*). These results suggest that the 0.5 M extract contains a factor(s) that specifically binds to an alphoid DNA.

Specific Interaction of Centromere Antigen with Alphoid DNA

To examine whether centromere antigens are involved in the interaction with alphoid DNA, the DNA-protein complexes were immunoprecipitated with anticentromere sera and the radioactive DNA fragments recovered were analyzed by agarose gel electrophoresis (Fig. 4). When 0.5 M NaCl extract was used, only α341 DNA fragment was recovered by immunoprecipitation (Fig. 4 *A*, lane *2*). On the other hand, incubation with 0.5–2 M NaCl extract led to precipitation of all DNA fragments at similar efficiencies (Fig. 4 *A*, lane *3*). To confirm that the precipitation of the alphoid DNA with the 0.5 M NaCl extract was dependent upon anticentromere antibody, other sera with or without anticentromere antibody were used for immunoprecipitation. All the sera from nine different autoimmune patients (A. K., S. T., H. M., R. K., T. M., K. G., H. K., Y., I. H.) carrying anticentromere antibody specifically precipitated α341 DNA fragment (Fig. 4 *B*, lanes *2–6* and data not shown), whereas the three normal sera (K., S., N.) did not precipitate any DNA fragments from the same preincubation mixture (lanes *7–9*). These results indicate that a centromere antigen present in the 0.5 M NaCl extract is involved in specific interaction with α341 alphoid DNA. Note that the amount of the precipitated α341 fragment was greatly reduced when a serum carrying the antibody for the 80-kD antigen in low level was used (Fig. 4 *B*, lane *6*).

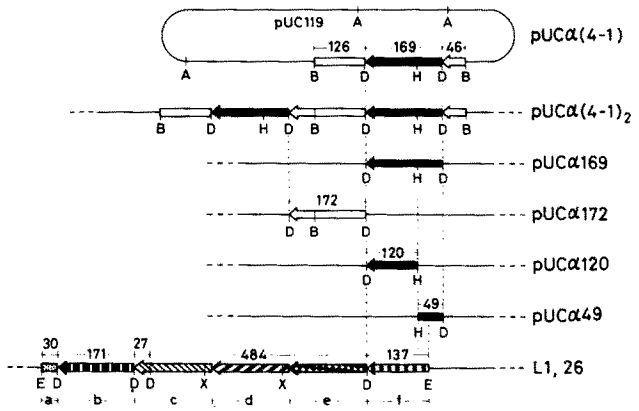

Figure 2. Structure of plasmid DNAs used. Inserts of alphoid DNA are shown as arrows and boxes and vector plasmid DNA is shown as lines. Plasmid pUCα(4-1) contains a single 341-bp alphoid Bam HI fragment. Plasmid pUCα$(4\text{-}1)_2$ contains two 341-bp alphoid Bam HI fragments tandemly. Full length arrows indicate complete alphoid DNA repeating units (170 bp). Shading of arrows indicates individual identities of alphoid repeating units in L1.26. Plasmids pUCα169, pUCα172, pUCα120, and pUCα49 contain subfragments of α(4-1) or $\alpha(4\text{-}1)_2$. L1.26 contains an 849-bp alphoid EcoRI fragment (Devilee et al., 1986). Restriction sites: *A*, Apa LI; *B*, Bam HI; *D*, Dde I; *H*, Hae III; *X*, Xba I. The numerals above the inserts indicate the nucleotides length (in basepairs) of the inserts. Small letters below the arrows of L1.26 identify each alphoid repeating unit.

When 0.25 M NaCl extract or 0.25–0.5 M NaCl extract was used, most of the activity for specific immunoprecipitation of alphoid DNA was found in 0.25–0.5 M NaCl extract (data not shown). Because the 80- and 140-, but not the 17-kD, centromere antigens were extracted between 0.25 and 0.5 M NaCl (Fig. 1 *B*), either one or both of these antigens may participate in the formation of immunoprecipitable complex with α341 DNA.

Region of Alphoid DNA Required for Interaction with Centromere Antigen

To examine the region of the α341 DNA that is required for formation of immunoprecipitable complex with factors present in 0.5 M extract, subfragments of the α341 DNA were tested. From the three subfragments of α341 DNA, namely α169, α126, and α46, which were generated by the digestion

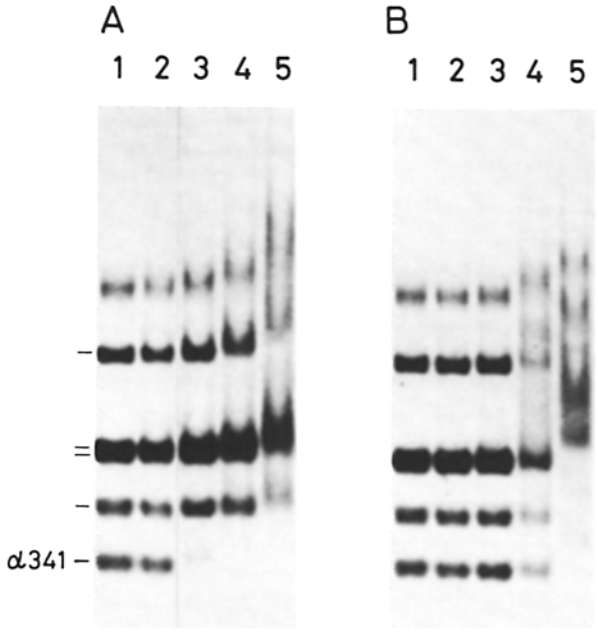

Figure 3. Detection of alphoid DNA-binding activity in nuclear-extracts using agarose gel. (*A*) ^{32}P-labeled Apa LI, Bam HI digests of plasmid pUCα(4-1) DNA (0.5 ng) were incubated with various amounts of 0.5 M NaCl extract in the presence of 2 μg poly(dI·dC) · poly(dI·dC) and then electrophoresed on a 1.5% agarose gel. Lane *1*, no extract added; lanes *2–5*, 0.25, 0.5, 1, and 2 μl of the extract added, respectively (1 μl of 0.5 M NaCl extract contained 6.2 μg protein derived from 1.3×10^5 nuclei). (*B*) Same as in *A*, except that 0.5–2 M NaCl extract and 0.5 μg of poly(dI·dC) · poly(dI·dC) were used. Lanes *1–5*, 0, 1, 2, 4, and 8 μl of 0.5–2 M NaCl extract was added, respectively (1 μl extract contained 2.5 μg protein derived from 1.3×10^5 nuclei). α341 indicates the 341-bp alphoid DNA fragment. Other vector-derived DNA fragments are indicated with a thin line adjacent to lane *1* in *A*.

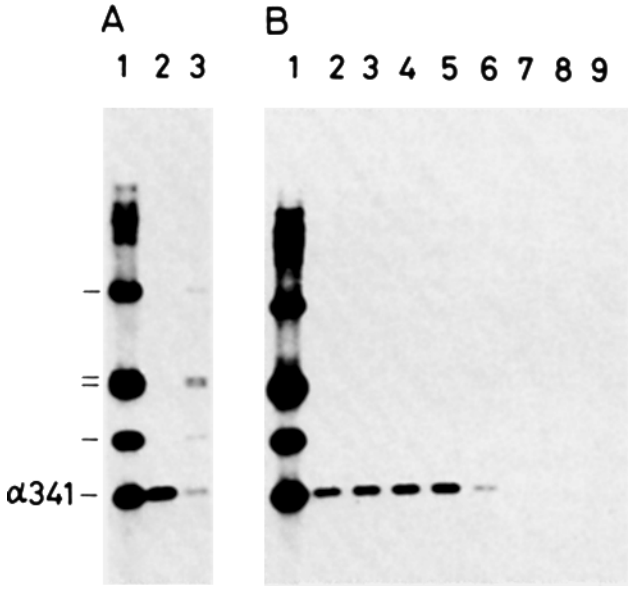

Figure 4. Immunoprecipitation of DNA fragments complexed with centromere antigen(s) in nuclear extracts. (*A*) ^{32}P-labeled Apa LI, Bam HI digests of plasmid pUCα(4-1) DNA (5 ng) and 5 μg of poly(dI·dC) · poly(dI·dC) were incubated with 0.5 M or 0.5–2 M NaCl extracts from HeLa nuclei. DNA-protein complexes were immunoprecipitated with anticentromere serum (A. K.) coupled to protein A-Sepharose. Immunoprecipitated DNA fragments were analyzed by electrophoresis on 1.5% agarose gel. Lane *1*, input DNA marker (75% radioactivity of the input was loaded); lane *2*, 3 μl (19 μg protein) of the 0.5 M NaCl extract. Lane *3*, 3 μl (7.5 μg protein) of the 0.5–2 M NaCl extract. (*B*) Same as in *A*, lane *2* except that various anticentromere sera and normal sera were used. Lane *1*, input DNA marker (75% of input amount); lanes *2–6*, anticentromere sera from patients, K. G., S. T., H. M., R. K., and T. M., respectively; lanes *7–9*, normal human sera, K., S., and N., respectively.

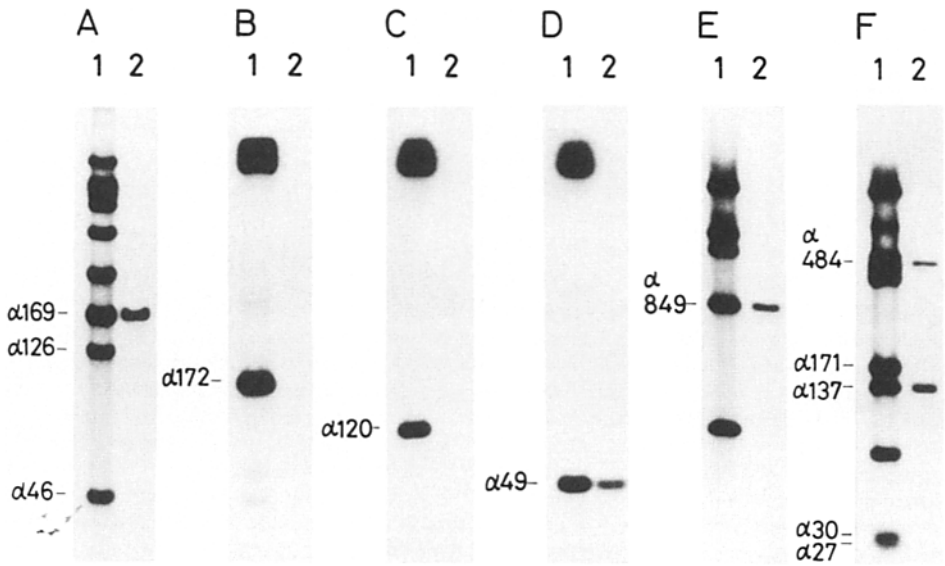

Figure 5. Immunoprecipitation of various subfragments of α341 and L1.26. Plasmids carrying α341 and L1.26 (see Fig. 2), digested with various restriction enzymes and ^{32}P-labeled, were incubated with 0.5 M NaCl extract and immunoprecipitated as described in Fig. 4 *A*, lane *2*. The precipitated DNA was analyzed by electrophoresis in either 8% polyacrylamide gels (*A*), 2.5% agarose gels (*B–D* and *F*) or 1.5% agarose gel (*E*). (*A*) pUCα(4-1) (digested with Bam HI/Dde I); (*B*) pUCα172 (Eco RI/Hind III); (*C*) pUCα 120 (Eco RI/Hind III); (*D*) pUCα 49 (Eco RI/Hind III); (*E*) L1.26 (Apa LI/Eco RI); (*F*) L1.26 (Dde I/Eco RI). Lane *1* in each panel, marker DNA (75% of input amount); lane *2* in each panel, immunoprecipitated DNA fragments. Numerals on the left side in each panel indicate nucleotide lengths of the fragments carrying alphoid DNA.

of pUCα(4-1) with Bam HI and Dde I (see Fig. 2), only α169 fragment was immunoprecipitated (Fig. 5 *A*). Next, plasmids carrying subfragment α172 (α126 plus α46), α120, or α49 (Fig. 2) were cleaved by Eco RI and Hind III at sites outside the cloned segment and used for immunoprecipitation assay. The results in Fig. 5 show that the α49 fragment, a subfragment of α169, was immunoprecipitated, whereas α172 or α120, another subfragment of α169, was not (Fig. 5, *B–D*). To compare the efficiency of formation of immunoprecipitable complex among the subfragments, nonradioactive plasmids carrying various fragments were used as competitors against the labeled α341 fragment. The plasmid carrying α341, α169, or α49 fragment inhibited the immunoprecipitation at a very similar efficiency to each other, whereas the plasmid carrying the α172 fragment or pUC119 itself did not (data not shown). These results indicate that the 49-bp fragment contains the region that is essential and sufficient for the formation of DNA-antigen complex.

To determine the boundary of the minimum region required to form the immunoprecipitable complex, duplex DNAs extended to each nucleotide position in the 49-bp region were made by primer extension on the single-strand template and used for the immunoprecipitation assay as described by Hawley-Nelson et al. (1988). The α169 was cloned into M13mp19 in both orientations (M13α169R and M13α169L). Standard dideoxy sequencing reactions were carried out on the single strand DNA of M13α169R or L, and the single-stranded region of the template was then digested with mung bean nuclease. Resulting duplex DNA fragments with different chain lengths were incubated with the 0.5 M NaCl extract, immunoprecipitated by the anticentromere serum and then analyzed by gel electrophoresis in a sequencing gel.

When M13 α169R was used as a template, molecules extended to and beyond the adenosine at position 265 (Masumoto et al., 1989) were all immunoprecipitated efficiently, whereas shorter molecules were not (Fig. 6, *2*). As for the M13 α169L template, molecules extended to and beyond the adenosine at position 251 were recovered by the immunoprecipitation assay (Fig. 6, *3*). Reduced efficiency of immuno-

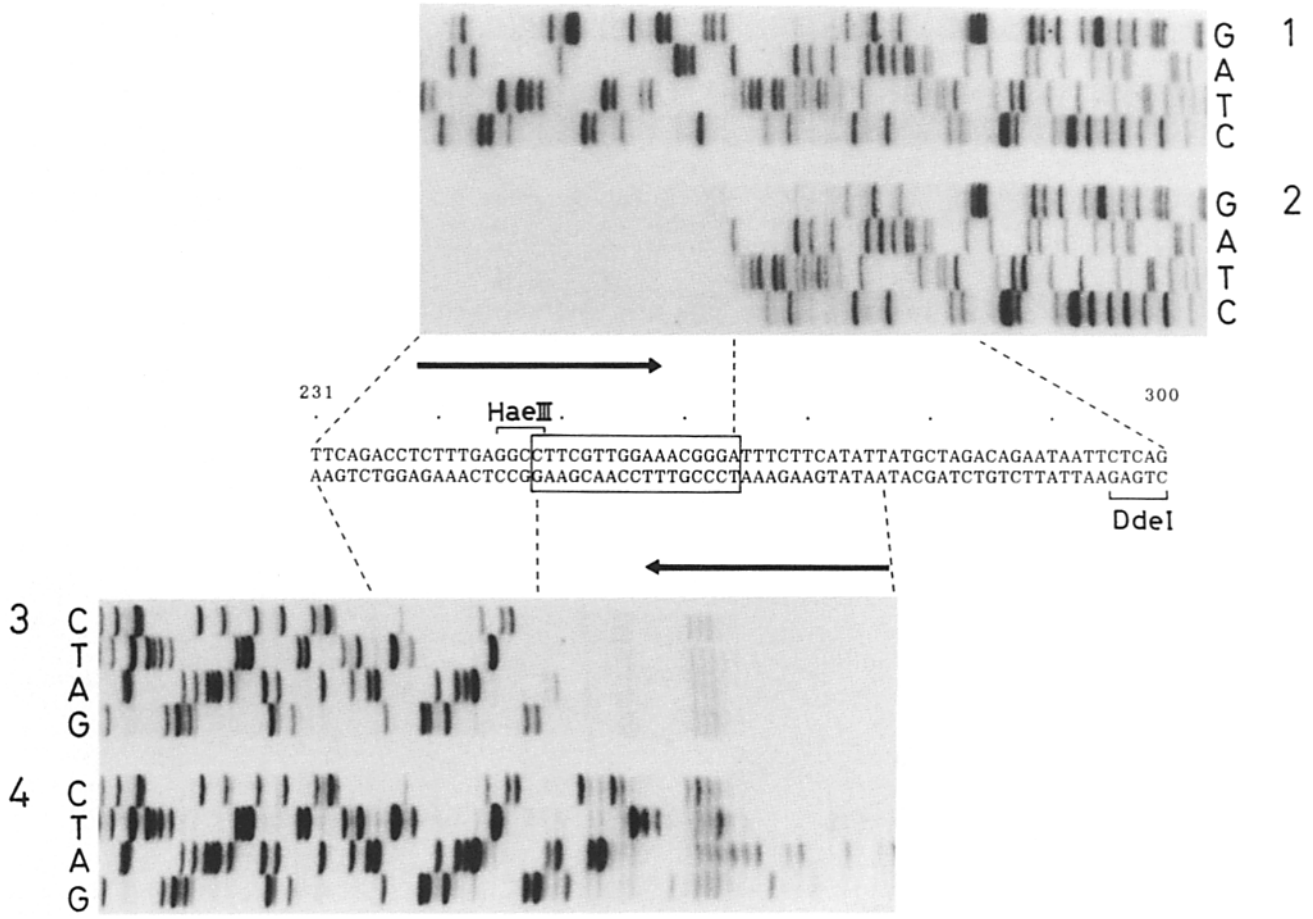

Figure 6. Determination of the minimum nucleotide sequence in α169 DNA required for DNA-antigen complex formation. After dideoxy sequencing reactions on single-stranded M13α169R or M13α169L template followed by digestion with mung bean nuclease, immunoprecipitation assays were carried out under the same conditions as in Fig. 4 *A*, lane *2*. Samples before and after immunoprecipitation were then analyzed by electrophoresis in polyacrylamide sequencing gels. Before (*1*) or after (*2*) immunoprecipitation of DNA using M13α169R electrophoresed in 6% polyacrylamide gel; before (*4*) or after (*3*) immunoprecipitation of DNA using M13α169L in 8% polyacrylamide gel. The arrows indicate the direction of primer elongation. The nucleotide sequence numbering corresponds to that in Fig. 3 of Masumoto et al. (1989). The box indicates the minimum nucleotide sequence required for efficient complex formation. The Hae III and Dde I sites are shown to demarcate the insert present in pUCα49 (see Fig. 2). Several abnormal ladders at around position 264 (*3* and *4*) were also precipitated with low efficiencies because of unknown reasons.

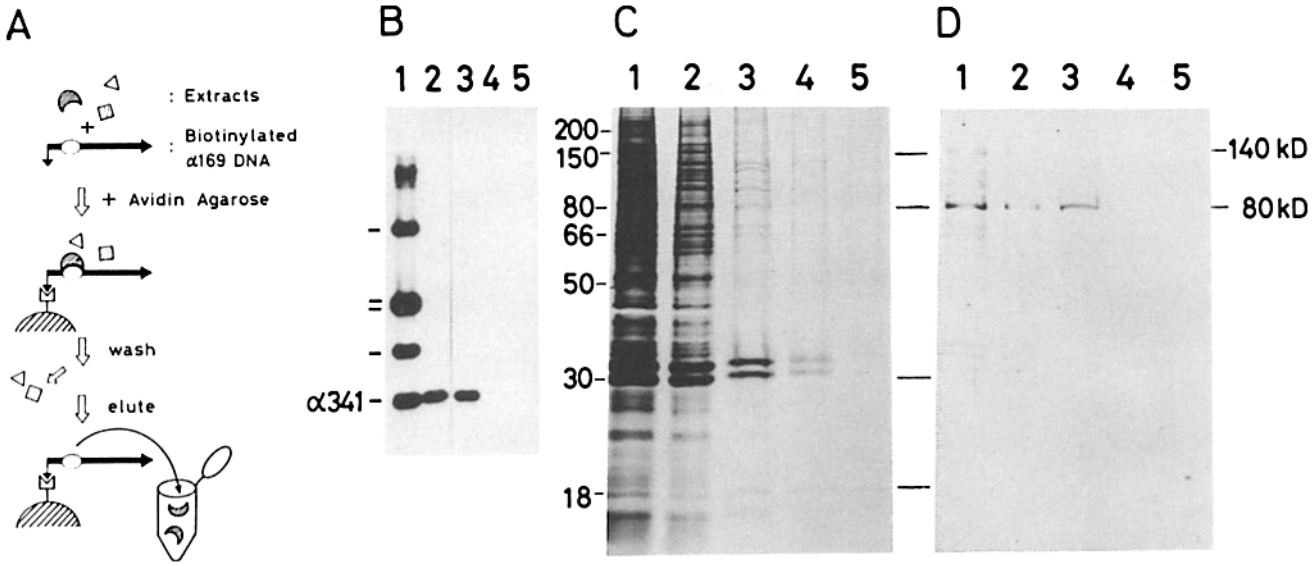

Figure 7. Identification of a centromere antigen bound to alphoid DNA. (*A*) Scheme for DNA affinity purification. Immunoprecipitable α169 DNA fragment and nonimmunoprecipitable control DNA were end-labeled with biotin-11-dUTP and [α-^{32}P]dCTP, and then incubated with a 0.25–0.5 M NaCl extract in the presence of poly(dI·dC) · poly(dI·dC). The biotinylated DNA fragments with the bound proteins were adsorbed with streptavidin-agarose beads, recovered by centrifugation and packed in columns. The recoveries of DNA fragments (α169, α172, and pUC), as monitored by ^{32}P radiolabel, were 67% (~9 × 10^{11} molecules), 62 and 77%, respectively. The columns were washed and bound proteins were eluted in buffer containing 0.5 M NaCl. (*B*) DNA immunoprecipitation assays were the same conditions as in Fig. 4 *A*, lane *2* except that 1.5 μl of 0.25–0.5 M NaCl extract or 0.5 μl (1/100 vol) of 0.5 M NaCl elute fractions from DNA affinity columns were used instead of 0.5 M NaCl extract. Lane *1*, marker DNA (75% of the input DNA was loaded); lane *2*, 0.25–0.5 M NaCl extract (4.3 μg protein from 2 × 10^5 nuclei); lane *3*, the eluate from the α169 DNA column (0.15 μg); lane *4*, the eluate from the α172 DNA column (0.15 μg); lane *5*, the eluate from the pUC119 DdeI 166 bp DNA column (0.06 μg). (*C*) Silver staining of recovered proteins. Proteins were electrophoresed on 10% polyacrylamide SDS gel, and silver stained. Lanes *1* and *2*, 3 and 1.5 μl, respectively, of 0.25–0.5 M NaCl extract; lane *3*, 10 μl of the 0.5 M NaCl eluate from the α 169 DNA column; lane *4*, 10 μl of the 0.5 M NaCl eluate from the α172 DNA column; lane *5*, 10 μl of the 0.5 M NaCl eluate from the pUC DNA column. Molecular weight standards are adjacent to the gel. (*D*) Immunoblotting. A duplicate gel of *C* was examined by immunoblotting with serum S. T.

precipitation for the molecules terminated at positions 250 and 251 suggest that DNA chains need to be extended to position 249 for efficient formation of immunoprecipitable complex.

Judged by these results, the 17-bp segment from position 249 through 265 (CTTCGTTGGAAACGGGA in the top strand in Fig. 6) is required for the efficient formation of the alphoid DNA-centromere antigen complex.

Sequences in Other Alphoid Families That Interact with Centromere Antigens

Because the α341 clone has been shown to hybridize with about one-third of human chromosomes (Masumoto et al., 1989), here we examined whether other alphoid clones that are not highly homologous to the α341 clone form the complex with centromere antigen(s). One of the plasmids used was L1.26, which carries an 849-bp alphoid DNA fragment (five 170-bp units) specific to chromosomes 13 and 21 (Fig. 2) (Devilee et al., 1986). Digestion of the plasmid with Eco RI and Dde I yielded five subfragments derived from α849 (484, 171, 137, 30, and 27 bp) (Fig. 2). The α849 fragment and the two out of five of its subfragments (α484 and α137) were recovered by the immunoprecipitation assay (Fig. 5, *E* and *F*). A similar experiment with Xho I-digested α484 fragment revealed that only the segment containing alphoid unit *d* (Fig. 2) was immunoprecipitable (data not shown). X chromosome–specific alphoid DNA, pXBR-1 (Yang et al., 1982), was also immunoprecipitated with anticentromere serum (data not shown).

Highly repeated nonalphoid sequences, the Alu I family, the Kpn I family, and satellite III sequences (Masumoto et al., 1989), were not recovered at all by the immunoprecipitation assay (data not shown).

Identification of the 80-kD Centromere Antigen in the Complex Formed with α169 DNA

To identify the centromere antigen(s) involved in complex formation on the alphoid DNA, proteins bound to α169 DNA were recovered from the crude reaction mixture, using biotinylated DNA and streptavidin agarose (Chodosh et al., 1986) as illustrated in Fig. 7 *A*. Three DNA fragments, immunoprecipitable α169 and two control fragments (α172 and a 166-bp fragment from the pUC vector) end-labeled with biotin-11-dUTP and [α-^{32}P]dCTP, were incubated with the 0.25–0.5 M NaCl extract. Streptavidin-agarose beads were added to each binding mixture and then DNA bound to beads were collected by centrifugation and packed into columns. Proteins bound were eluted with a buffer containing 0.5 M NaCl. First, we tested the activity of each eluate to bind to α341 DNA by immunoprecipitation. As seen in Fig. 7 *B*, α341 DNA was immunoprecipitated only when preincubated with the eluate from the α169 DNA column (lane *3*). Activity recovered from the α169 DNA column was roughly estimated to be 7% of the input, whereas protein recovery was 0.3%. Then, proteins in each eluate were fractionated by SDS-PAGE, and analyzed by silver staining and by immunoblotting with the anticentromere serum. As visualized by sil-

ver staining, the α169 sample contained a larger amount of proteins with various molecular weights than other samples did (Fig. 7 *C*). By immunoblotting, the 80-kD centromere antigen was found in the fraction eluted from the α169 DNA column (Fig. 7 *D*, lane *3*), whereas no antigen was detected in the corresponding fraction from either α172 (lane *4*) or pUC (lane *5*) DNA columns. The 140-kD centromere antigen was not found in any eluates.

Results that the 80-kD centromere antigen is present only in the eluate from α169 DNA and that only this eluate has the ability to form an immunoprecipitable complex with α341 DNA suggest that the 80-kD centromere antigen plays a role in formation of α169 DNA-specific antigenic complex.

Binding of the 80-kD Antigen to the DNA Fragment Carrying the 17-bp Motif

The results described above led us to examine whether the 80-kD centromere antigen itself binds to DNA fragments carrying the 17-bp motif that is required for formation of immunoprecipitable complex. Proteins in 0.5 M NaCl extract or those precipitated from the extract with the anticentromere antibody (I. H.) conjugated to Sepharose beads were fractionated by SDS-PAGE and transferred to the IPVH membrane. DNA binding activity of the membrane-bound proteins was probed with ^{32}P-end-labeled 56-bp DNA containing the 17-bp recognition motif. When the 0.5 M NaCl extract was used, the labeled probe DNA bound to many positions, including one that had a similar mobility with the 80-kD antigen (Fig. 8, lane *5*). On the other hand, with the sample precipitated with the antibody-linked Sepharose beads, the probe preferentially bound to the polypeptide that comigrated with the 80-kD centromere antigen, although weak DNA binding activity was also detected at several other positions (Fig. 8, lanes *1* and *2*). The binding of radioactive DNA to membrane was completely competed out by 100-fold excess of the nonradioactive 56-bp DNA (lane *3*). In contrast, addition of 400-fold excess of poly(dI·dC)·poly(dI·dC) to the incubation mixture did not affect the intensity of the band at the 80-kD polypeptide, while weak bands at other positions disappeared (lane *4*). The results, that the 80-kD polypeptide precipitated by centromere antibody specifically binds to the 56-bp DNA fragment, strongly suggest that the 80-kD centromere antigen itself has activity to bind the DNA carrying the 17-bp motif.

Discussion

In this study, we have reported the molecular interaction between centromere antigens in HeLa nuclear extract and subclasses of alphoid DNA monomers. A centromere antigen recognizes a distinct region of a certain class of alphoid DNA and forms a DNA-antigen complex that is selectively immunoprecipitable. These results provide the molecular basis of our previous cytological observations that sites of the alphoid DNA repeat in the human chromosomes perfectly overlap with the sites of centromere antigens throughout the cell cycle (Masumoto et al., 1989). The in vivo and in vitro results together suggest a possibility that alphoid DNA plays some crucial role in centromere and/or kinetochore in human chromosomes through the interaction with a centromere antigen.

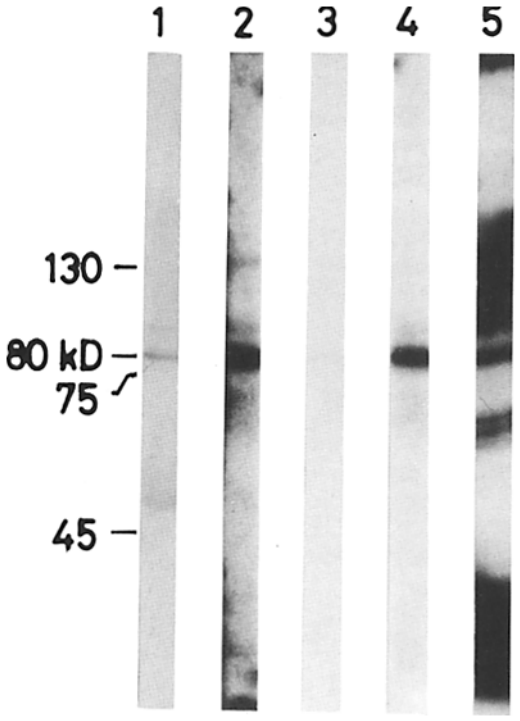

Figure 8. Detection of selective binding of the DNA fragment carrying the 17-bp motif to the 80-kD centromere antigen. Proteins in the 0.5 M NaCl extract (lane *5*, 1.6 × 10^6 nuclei) and those of affinity precipitated from the extract using anticentromere antibodies conjugated to Sepharose beads (lanes *1–4*, 8 × 10^6 nuclei/lane) were electrophoresed on 7.5% polyacrylamide gel containing SDS, transferred to PVDF membrane, and then probed with an anticentromere serum (lane *1*) and the ^{32}P-labeled 56-bp DNA carrying the 17-bp motif (10^6 cpm/27 ng/lane) (lanes *2–5*). Lane *1*, centromere antigen detected by immunoblotting with serum I. H. A band at the 50-kD position is IgG heavy chain coeluted from the Sepharose beads after the immunoprecipitation. Lanes *2* and *5*, radioactive bands detected without competitor DNA; lane *3*, addition of nonradioactive 56-bp DNA (2.7 μg/ml) to the reaction in lane *2*; lane *4*, addition of poly(dI·dC) · poly(dI·dC) (10 μg/ml) to the reaction in lane *2*. Positions for molecular weight marker proteins are indicated on the left of lane *1*.

Extraction of Centromere Antigens from Nuclei of HeLa Cells

We have shown in this study that the majority of 80- and 140-kD centromere antigen is extracted from the HeLa cell nuclei by 0.25–0.5 M NaCl buffer, whereas about a half portion of the 17-kD antigen is extracted together with major histones by 0.5 M–2 M NaCl and the rest of the antigen remains in the nuclear pellet (Fig. 1). Earlier reports from other laboratories, however, indicated that the 80-kD antigen was tightly associated with the histone-depleted scaffold of metaphase chromosome produced by micrococcal nuclease digestion followed by histone extraction with either polyanions (dextran sulfate/heparin) or 2 M NaCl (Earnshaw et al., 1984; Valdivia and Brinkley, 1985). 17-kD antigen was released together with histones from nuclei by the micrococcal nuclease digestion followed by the heparin treatment (Valdivia and Brinkley, 1985). In this study, the 80-kD antigen was released from the nuclei in relatively low concentrations of

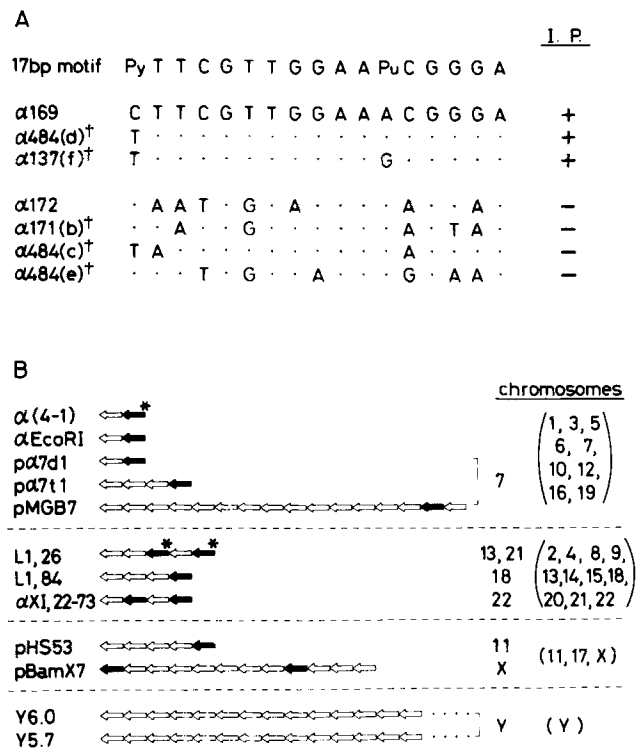

Figure 9. A consensus CENP-B box and its distribution in various alphoid DNA clones. (*A*) 17-bp recognition sequence defined by experiments in Fig. 6 and corresponding regions of immunoprecipitable and nonimmunoprecipitable alphoid monomers. †Alphoid repeating units of L1.26 as shown in Fig. 2. The results of immunoprecipitation (Fig. 5 and data not shown) are indicated by + (precipitated) or − (not precipitated). (*B*) Distribution of the CENP-B box in various alphoid DNA clones. Black or white arrows indicate alphoid monomers with or without consensus sequence of the CENP-B box, respectively. Chromosome number from which alphoid DNA clones were derived is indicated in the column on the right. Numerals in parentheses indicate chromosome numbers to which alphoid DNA groups were located by in situ hybridization (Alexandrov et al., 1988). α Eco RI indicates a consensus sequence of alphoid Eco RI dimer (Wu and Manuelidis, 1980); pα 7dl, pα 7tl, pMGB7 (Waye et al., 1987); pBam X7 (Waye and Willard, 1985); L1.26 and L1.84 (Devilee et al., 1986); α XI, 22-73 (Jørgensen et al., 1987); pHS 53 (Alexandrov et al., 1988); Y6.0 and Y5.7 indicate 6.0- and 5.7-kb alphoid DNA clones, respectively, from the Y chromosome (Tyler-Smith and Brown, 1987). *Alphoid DNA units shown to be immunoprecipitable by this work.

NaCl solution. This might indicate that the 80-kD antigen in interphase nuclei has a different property from that in metaphase chromosomes and thus is loosely associated with the pre-kinetochore structure. Alternatively, our extraction method of nuclear proteins, which includes no metal ion–requiring steps (for example, micrococcal nuclease digestion in the presence of $CaCl_2$) and uses extraction buffers containing metal chelator (EDTA) and thiol reagent (DTT) at 4°C, might be more efficient for dissociation of the antigens from the chromatin than the condition used by previous workers (Lewis and Laemmli, 1982).

Nucleotide Sequence of Alphoid DNA Required for Interaction with a Centromere Antigen

The gel shift assays show that the 0.5 M NaCl HeLa nuclear extract contains a factor(s) that specifically binds to α341 fragments (alphoid dimers) (Fig. 3). Involvement of centromere antigens in the α341 specific complex is demonstrated by the selective precipitation with anticentromere sera of α341 DNA from the mixture of DNA fragments preincubated with the 0.5 M NaCl nuclear extract (Fig. 4). The immunoprecipitable α341 DNA is composed of two tandem alphoid monomers, α169 and α172, 75% homologous with each other. The region required to form the antigenic complex with the 0.5 M NaCl extract resides within the 49-bp segment of the α169 (Fig. 5 *D*). The minimum essential sequence required for the formation of DNA-antigen complex has been defined as a 17-bp segment within the 49-bp region of α169 by immunoprecipitation of primer extension products (Fig. 6). The corresponding region in the nonimmunoprecipitable α172 DNA contained a sequence different at seven nucleotides from this motif (Fig. 9 *A*). The DNA-immunoprecipitation assay with another alphoid clone (L1.26 derived from chromosomes 13 and 21), which has relatively low sequence homology to α169 (Devilee et al., 1986), has revealed that each of two immunoprecipitable segments of L1.26 contains a motif that matches 16 out of 17 bp (unit *d*) and 15 out of 17 bp (unit *f*), whereas the segments not immunoprecipitable (units *b*, *c*, and *e*) have motifs differing >3 bp (Figs. 2 and 9 *A*). The consensus sequence of the 17-bp motif in three immunoprecipitable alphoids is shown in Fig. 9. The 17-bp motif is relatively GC rich compared with other regions of alphoid DNA, and internally is pyrimidine rich toward its 5′ side and purine rich toward its 3′ side. A short internal inverted repeat is present in the middle of the motif containing a CG stretch at each end (CGTTGGAAPuCG).

Alphoid sequences have been classified into several subclasses based on the results of in situ hybridization (Alexandrov et al., 1988). A computer analysis of known alphoid sequences revealed that the 17-bp motif is present in various subclasses of alphoid covering almost all chromosomes, despite extensive divergence of the nucleotide sequences between subclasses (Fig. 9 *B*). The frequency of alphoid monomer carrying the 17-bp motif appears to be very different even among clones derived from the same chromosome. For example, each of three clones derived from chromosome 7 (Waye et al., 1987) contains the 17-bp motif in every 2, 4, or 16 repeating units, respectively (Fig. 9 *B*). Clones of alphoid derived from the Y chromosome (Tyler-Smith and Brown, 1987) do not contain the motif. At present, we do not know whether the 17-bp motif is not present in the alphoid of the Y chromosome, or the cloned alphoid sequences are derived from the region where the frequency of the motif is low. The presence of the 17-bp motif in the centromere region of all chromosomes, so far with exception of the Y chromosome, suggests that the motif has some important role in the function of the alphoid DNA in the centromere region.

It is of interest to ask whether the centromere regions of other mammalian chromosomes have a similar motif. In the mouse chromosome, the major and the minor satellite sequences are known as constituents of centromeric heterochromatin (Pietras et al., 1989). In the major satellite sequence, which forms a main part of the heterochromatin, no sequence similar to the 17-bp motif is found. On the other hand, a motif that differs one nucleotide from the 17-bp motif (ATTCGTTGGAAACGGGA) is present in the consensus

sequence of the minor satellite repeat which was found in all the centromeric regions of *Mus musculus* chromosomes (Wong and Rattner, 1988). The location of the minor satellite is assigned at or immediately adjacent to the kinetochore on the mouse chromosome which was detected by anticentromere/kinetochore sera of human autoimmune patient (Wong and Rattner, 1988).

The 80-kD Centromere Antigen (CENP-B) Recognizes the 17-bp Motif, the CENP-B Box

The anticentromere sera we used recognize three major antigens in common. Among them, both 80- and 140-kD antigens are present in the 0.5 M NaCl HeLa nuclear extract that contains a factor(s) that specifically interacts with the alphoid DNA carrying the 17-bp motif. Independent lines of evidence listed below suggest that the 80-kD antigen (CENP-B) is a factor that forms a complex with the DNA carrying the motif. First, when we recovered the nuclear proteins bound to α169 DNA, the fraction, which retained the ability to form antigenic complex with α341 DNA, contained the 80-kD centromere antigen (CENP-B) but not the 140-kD centromere antigen (Fig. 7). Second, an anticentromere serum with very low antibody activity against the 80-kD antigen precipitated the α341 DNA with a decreased efficiency (Fig. 4 *B*, lane *6*). Third, when HeLa nuclear proteins immunoprecipitated with anticentromere serum were separated by electrophoresis and immobilized on a PVDF membrane, the activity to bind specifically to the DNA carrying the 17-bp motif was detected at the same position as the 80-kD antigen. The most likely interpretation of these results is that the 80-kD antigen itself binds to the DNA carrying the 17-bp motif. Therefore, we refer to the 17-bp motif that is required for the recognition by the 80-kD antigen (CENP-B) as CENP-B box.

A cDNA clone covering >90% of the coding region of CENP-B has been isolated and the nucleotide sequence of the cloned segment has been determined (Earnshaw et al., 1987). The carboxyl-terminus of the predicted polypeptide contains two very highly acidic domains. The authors suggested that these domains may interact with basic domains of other chromatin proteins. It should be noted that the affinity-purified fraction from the α169 DNA contains several polypeptides more abundantly than the fraction recovered from the control DNAs (Fig. 7 *C*). It remains to be solved whether some of these proteins have affinity to the 80-kD centromere antigen and play a role in the structure and function of the centromere-kinetochore region. No interactions have yet been detected among the three centromere antigens; the 0.5–2 M NaCl nuclear extract did not affect the specific immunoprecipitation of the α341 DNA by the 80-kD antigen (data not shown), or the 140-kD antigen was not recovered from the complex made on the DNA carrying the CENP-B box (Fig. 7).

Solomon et al. (1986) reported that a high mobility group–like nuclear protein (α-protein) from African green monkey cells preferentially binds at three AT rich sites of 172-bp α-satellite DNA (the counterpart of human alphoid DNA). Properties of the α-protein are very different from those of the 80-kD centromere antigen, as it binds to any run of six or more AT basepairs and co-migrates with the HMG-17 by electrophoresis in an SDS gel (Strauss and Varshavsky, 1984).

Kinetochores on chromosomes are the attachment sites of spindle microtubules during meiosis and mitosis. Several observations suggest that chromatin is a basic component of higher ordered kinetochore structure (Ris and Witt, 1981; Rattner, 1987). Our previous results suggest tight association of centromere antigens to alphoid DNA repeats at centromere regions of chromosomes even in chromosomes artificially decondensed and stretched by hypotonic treatment followed by centrifugation (Masumoto et al., 1989). We now have shown that certain alphoid DNA repeats indeed have recognition sequences (CENP-B box) for the interaction with 80-kD centromere antigens. Because the 80-kD antigen is a tightly associated component of kinetochore (Valdivia and Brinkley, 1985), the alphoid DNA itself is likely to be a basic component of kinetochore. The interaction of the CENP-B box in the long tandem repeat of alphoid sequence with the 80-kD antigen might have an important role in the process of the formation of specific centromere structure and in the assembly of a kinetochore.

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References

Alexandrov, I. A., S. P. Mitkevich, and Y. B. Yurov. 1988. The phylogeny of human chromosome specific alpha satellites. *Chromosoma (Berl.).* 96: 443–453.

Beridze, T. 1986. Satellite DNA. Springer-Verlag, Berlin. 1–149.

Berman, J., C. Y. Tachibana, and B.-K. Tye. 1986. Identification of a telomere-binding activity from yeast. *Proc. Natl. Acad. Sci. USA.* 83:3713–3717.

Brenner, S., D. Pepper, M. W. Berns, E. Tan, and B. R. Brinkley. 1981. Kinetochore structure, duplication, and distribution in mammalian cells: analysis by human autoantibodies from scleroderma patients. *J. Cell Biol.* 91:95–102.

Celenza, J. L., and M. Carlson. 1986. A yeast gene that is essential for release from glucose repression encodes a protein kinase. *Science (Wash. DC).* 233:1175–1180.

Chodosh, L. A., R. W. Carthew, and P. A. Sharp. 1986. A single polypeptide possesses the binding and transcription activities of the adenovirus major late transcription factor. *Mol. Cell. Biol.* 6:4723–4733.

Clarke, L., and J. Carbon. 1980. Isolation of a yeast centromere and construction of functional small circular chromosomes. *Nature (Lond.).* 287:504–509.

Clarke, L., and J. Carbon. 1985. The structure and function of yeast centromeres. *Annu. Rev. Genet.* 19:29–56.

Comings, D. E., and T. A. Okada. 1971. Fine structure of kinetochore in indian muntjac. *Exp. Cell Res.* 67:97–110.

Devilee, P., P. Slagboom, C. J. Cornelisse, and P. L. Pearson. 1986. Sequence heterogeneity within the human alphoid repetitive DNA family. *Nucleic Acids Res.* 14:2059–2073.

Earnshaw, W. C., N. Halligan, C. Cooke, and N. Rothfield. 1984. The kinetochore is part of the metaphase chromosome scaffold. *J. Cell Biol.* 98:352–357.

Earnshaw, W. C., and N. Rothfield. 1985. Identification of a family of human centromere proteins using autoimmune sera from patients with scleroderma. *Chromosoma (Berl.).* 91:313–321.

Earnshaw, W. C., K. F. Sullivan, P. S. Machlin, C. A. Cooke, D. A. Kaiser, T. D. Pollard, N. F. Rothfield, and D. W. Cleveland. 1987. Molecular cloning of cDNA for CENP-B, the major human centromere autoantigen. *J. Cell Biol.* 104:817–829.

Hahnenberger, K. M., M. P. Baum, C. M. Polizzi, J. Carbon, and L. Clarke. 1989. Construction of functional artificial minichromosomes in the fission yeast *Schizosaccaromyces pombe*. *Proc. Natl. Acad. Sci. USA.* 86:577–581.

Hawley-Nelson, P., E. J. Androphy, D. R. Lowy, and J. T. Schiller. 1988. The specific DNA recognition sequence of the bovine papillomavirus E2 protein is an E2-dependent enhancer. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:525–531.

Hieter, P., D. Pridmore, J. H. Hegemann, M. Thomas, R. W. Davis, and P. Philippsen. 1985. Functional selection and analysis of yeast centromeric DNA. *Cell.* 42:913–921.

Johnstone, A., and R. Thorpe. 1982. Immunochemistry in Practice. Blackwell Scientific Publications, Oxford. 298 pp.

Jokelainen, P. T. 1967. The ultrastructure and spatial organization of the metaphase kinetochore in mitotic rat cells. *J. Ultrastruct. Res.* 19:19–44.

Jones, K. W., and G. Corneo. 1971. Location of satellite and homogeneous DNA sequences on human chromosomes. *Nat. New Biol.* 233:268–271.

Jørgensen, A. L., C. J. Bostock, and A. L. Bak. 1987. Homoglous subfamilies of human alphoid repetitive DNA on different nucleolus organizing chromosomes. *Proc. Natl. Acad. Sci. USA.* 84:1075–1079.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Lewis, C. D., and U. K. Laemmli. 1982. Higher order metaphase chromosome structure: Evidence for metalloprotein interactions. *Cell.* 29:171–181.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 75–96.

Manuelidis, L. 1976. Repeating restriction fragments of human DNA. *Nucleic Acids Res.* 3:3063–3076.

Manuelidis, L. 1978. Chromosomal localization of complex and simple repeated human DNAs. *Chromosoma (Berl.).* 66:23–32.

McGrew, J., B. Diehl, and M. Fitzgerald-Hayes. 1986. Single base pair mutations in centromere element III cause aberrant chromosome segregation in *Saccharomyces cerevisiae. Mol. Cell. Biol.* 6:530–538.

Masumoto, H., K. Sugimoto, and T. Okazaki. 1989. Alphoid satellite DNA is tightly associated with centromere antigens in human chromosomes throughout the cell cycle. *Exp. Cell Res.* 181:181–196.

McKay, R. D. G. 1981. Binding of a simian virus 40 T antigen-related protein to DNA. *J. Mol. Biol.* 145:471–488.

Moroi, Y., C. Peebles, M. J. Fritzler, J. Steigerwald, and E. M. Tan. 1980. Autoantibody to centromere (kinetochore) in scleroderma sera. *Proc. Natl. Acad. Sci. USA.* 77:1627–1631.

Moroi, Y., A. L. Hartman, P. K. Nakane, and E. M. Tan. 1981. Distribution of kinetochore (centromere) antigen in mammalian cell nuclei. *J. Cell Biol.* 90:254–259.

Ng, R., and J. Carbon. 1987. Mutational and in vitro protein-binding studies on centromere DNA from *Saccharomyces cerevisiae. Mol. Cell. Biol.* 7: 4522–4534.

Palmer, D. K., K. O'Day, M. H. Wener, B. S. Andrews, and R. L. Margolis. 1987. A 17-kD centromere protein (CENP-A) copurifies with nucleosome core particles and with histones. *J. Cell Biol.* 104:805–815.

Pardue, M. L., and J. G. Gall. 1970. Chromosomal localization of mouse satellite DNA. *Science (Wash. DC).* 168:1356–1358.

Pietras, D. F., K. L. Bennett, L. D. Siracusa, M. Woodworth-Gutai, V. M. Chapman, K. W. Gross, C. Kane-Haas, and N. D. Hastie. 1983. Construction of a small *Mus musculus* repetitive DNA library: identification of a new satellite sequence in *Mus musculus. Nucleic Acids Res.* 11:6965–6983.

Rattner, J. B. 1987. The organization of the mammalian kinetochore: a scanning electron microscope study. *Chromosoma (Berl.).* 95:175–181.

Rieder, C. L. 1982. The formation, structure, and composition of the mammalian kinetochore and kinetochore fiber. *Int. Rev. Cytol.* 79:1–58.

Ris, H., and P. L. Witt. 1981. Structure of the mammalian kinetochore. *Chromosoma (Berl.).* 82:153–170.

Roos, U.-P. 1973. Light and electron microscopy of rat kangaroo cells in mitosis. II. Kinetochore structure and function. *Chromosoma (Berl.).* 41:195–220.

Saunders, M., M. Fitzgerald-Hayes, and K. Bloom. 1988. Chromatin structure of altered yeast centromeres. *Proc. Natl. Acad. Sci. USA.* 85:175–179.

Solomon, M. J., F. Strauss, and A. Varshavsky. 1986. A mammalian high mobility group protein recognizes any stretch of six A·T base pairs in duplex DNA. *Proc. Nalt. Acad. Sci. USA.* 83:1276–1280.

Strauss, F., and A. Varshavsky. 1984. A protein binds to a satellite DNA repeat at three specific sites that would be brought into mutual proximity by DNA folding in the nucleosome. *Cell.* 37:889–901.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

Tyler-Smith, C., and W. R. A. Brown. 1987. Structure of the major block of alphoid satellite DNA on the human Y chromosome. *J. Mol. Biol.* 195: 457–470.

Valdivia, M. M., and B. R. Brinkley. 1985. Fractionation and initial characterization of the kinetochore from mammalian metaphase chromosomes. *J. Cell Biol.* 101:1124–1134.

Vieira, J., and J. Messing. 1987. Production of single-stranded plasmid DNA. *Methods Enzymol.* 153:3–11.

Waye, J. S., S. B. England, and H. F. Willard. 1987. Genomic organization of alpha satellite DNA on human chromosome 7: evidence for two distinct alphoid domains on a single chromosome. *Mol. Cell. Biol.* 7:349–356.

Waye, J. S., and H. F. Willard. 1985. Chromosome-specific alpha satellite DNA: nucleotide sequence analysis of the 2.0 kilobasepair repeat from the human X chromosome. *Nucleic Acids Res.* 13:2731–2743.

Willard, H. F., and J. S. Waye. 1987. Hierarchical order in chromosome-specific human alpha satellite DNA. *Trends Genet.* 3:192–198.

Wong, A. K. C., and J. B. Rattner. 1988. Sequence organization and cytological localization of the minor satellite of mouse. *Nucleic Acids Res.* 16:11645–11661.

Wu, J. C., and L. Manuelidis. 1980. Sequence definition and organization of a human repeated DNA. *J. Mol. Biol.* 142:363–386.

Yang, T. P., S. K. Hansen, K. K. Oishi, O. A. Ryder, and B. A. Hamkalo. 1982. Characterization of a cloned repetitive DNA sequence concentrated on the human X chromosome. *Proc. Natl. Acad. Sci. USA.* 79:6593–6597.