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Biochemistry

A 47-kDa human nuclear protein recognized by antikinetochore autoimmune sera is homologous with the protein encoded by *RCC1*, a gene implicated in onset of chromosome condensation

(amino acid sequence/autoantigen/mitosis/posttranslational modification/CREST syndrome)

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ABSTRACT **Several autoimmune sera from patients with Raynaud phenomenon decorated mammalian kinetochores and bound to a 47-kDa protein on immunoblots of nuclear lysates. Antibody affinity-purified from immunoblots of the 47-kDa band recognized kinetochores, but due to crossreaction with an 18-kDa protein, localization remains elusive. We used one of these sera to purify the antigen from HeLa cells synchronized in mitosis as a noncovalent complex with a 25-kDa protein. The antigen was released from DNA by intercalation with 25 mM chloroquine. Ion-exchange chromatography yielded the pure complex with an apparent molecular size of 68 kDa, which was separated into its components by gel filtration in 6 M guanidinium chloride. Upon two-dimensional gel electrophoresis the 47-kDa protein gave two main spots of pI 6.6 and 6.7, respectively. Posttranslational modification is indicated by additional antigenic spots, by lack of a free α-amino group, and by chromatographic behavior of peptides on reversed-phase chromatography. The amino acid sequence for 205 residues of the 47-kDa protein has been established. This sequence is highly homologous with the translated reading frame of *RCC1*, a gene reportedly involved in regulating onset of mammalian chromosome condensation.**

Kinetochores are the sites of chromosome attachment to the fibers of the mitotic spindle. In electron micrographs, a trilaminar structure embedded in the chromatin (1–3) is frequently seen to interact with microtubules nucleated from the spindle poles either laterally or with their "plus" ends (4). Poleward force is exerted on the chromosomes, and current models imply that the kinetochores represent a motor for steadily moving separated chromatids toward the poles during anaphase (5, 6). This structure is the target of a highly selective autoimmune response in patients with Raynaud phenomenon (7–9), a disease of unknown etiology frequently associated with the CREST (calcinosis, Raynaud's phenomenon, esophageal hypomotility, sclerodactyly, telangiectasia) form of scleroderma. By using autoimmune sera, various proteins associated with this structure have been identified as bands on immunoblots (10–15). Crosslinking experiments suggest that a set of proteins with molecular masses of 110-, 80-, 54-, and 24-kDa can bind microtubules (16). Other components bind to specific centromere DNA sequences (17, 18). The 80-kDa protein has been cloned and sequenced (19).

A 47-kDa protein is recognized by 11 of 44 kinetochore-staining sera in our collection. Here we describe the isolation and characterization of this protein using one of our sera and discuss its homology with RCC1 protein, a regulator of the onset of chromosome condensation.

MATERIALS AND METHODS

Indirect Immunofluorescence. Fibroblast cells of the Indian muntjac (*Muntiacus muntjae*) (from K. Sperling, Freie Universität Berlin) and adherent HeLa cells (W. W. Franke, Heidelberg) were grown in monolayer cultures on multitest slides (Flow Laboratories) in Eagle's minimal essential medium/Earle's medium supplemented with 10% fetal calf serum. Cells were fixed for 10 min in 3.7% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS) at 37°C. After being washed three times in PBS, the cells were permeabilized in methanol/acetone, 3:1 (vol/vol) for 3 min at −20°C. The slides were rinsed in PBS and incubated for 1 hr at 37°C with 1:500 dilutions of the autoimmune serum. Excess antibody was removed in three washes with PBS/0.01% Tween 20. After incubation with fluorescein-conjugated anti-human immunoglobulin (H+L; from Dianova, Hamburg) diluted 1:100, the cells were washed twice in PBS/0.01% Tween and once in distilled water. They were then mounted in Mowiol (Calbiochem) containing *p*-phenylenediamine at 1 mg/ml and observed under a Zeiss IM 35 microscope equipped with filters BP 450-490 and LP 520.

Growth and Synchronization of HeLa Cells. HeLa S3 suspension cells (from W. Keller, Heidelberg) were grown in large Erlenmeyer flasks in Joklik's modified Eagle's minimal essential medium supplemented with 5% newborn calf serum (Biochrom, Berlin). For synchronization in mitosis, nocodazole at 0.08 μg/ml (Janssen Pharmaceutica) was added to the medium for 20 hr, yielding a mitotic index of >90%. Cells were collected by centrifugation at 800 × *g* and washed twice in PBS containing nocodazole at 0.08 μg/ml and once in PBS containing protease inhibitors. Cells (≈10^9/ml) were stored at −80°C. Alternatively, logarithmically growing unsynchronized cultures were used for some experiments.

Immunoblots. After electrophoresis in SDS on 12% gels, proteins were transferred to nitrocellulose (Schleicher & Schuell) according to Towbin *et al.* (20) with 0.001% SDS in the transfer buffer. Blots were stained with Ponceau S (Sigma), destained with TBS (10 mM Tris·HCl, pH 7.6/150 mM NaCl), blocked for 15 min with 5% hydrolyzed gelatin in TBS, rinsed in the same buffer, and incubated with the autoimmune serum (diluted 1:300 in TBS/0.5% gelatin) for 1 hr at room temperature. After five washes with TBS/0.1% Tween 20 and one with TBS, biotinylated sheep anti-human immunoglobulin (Amersham) diluted 1:400 in TBS/gelatin was added for 1 hr. The sheet was washed as above, incubated for 30 min with a 1:400 dilution of streptavidin-biotinylated horseradish peroxidase complex (Amersham) in TBS/gelatin and again washed. Bound antibodies were de-

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tected by using chloronaphthol [9 ml of 0.3% 4-chloro-1-naphthol in methanol, 75 ml of TBS, and 30 μl of 30% (vol/vol) H_2O_2] as substrate for peroxidase.

Autoimmune Serum and Affinity Purification of Autoantibodies. The serum used for antigen isolation was obtained from a female patient of 45 yr with Raynaud phenomenon but devoid of other symptoms of scleroderma. Autoantibodies bound to specific polypeptide bands immobilized on nitrocellulose were eluted by incubation with 0.1 M glycine/0.1 M NaCl/0.5% bovine serum albumin, pH 2.5, for 3 min. The eluent was immediately neutralized with 1 M Tris, and autoantibodies were tested by immunofluorescence on HeLa cells and by staining blots of HeLa cell total proteins.

Protease Inhibitors. A stock solution in methanol was prepared of 0.5 mM phenylmethanesulfonyl fluoride/0.1 mM tosyl-L-lysine chloromethyl ketone/0.2 mM tosyl-L-phenylalanine chloromethyl ketone/leupeptin at 0.5 mg/liter/pepstatin A at 0.7 mg/liter/0.2 mM N^{α}-*p*-tosyl-L-arginine methyl ester/E 64 {*N*-[*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine} at 0.5 mg/liter/phosphoramidon at 10 mg/liter/bestatin at 40 mg/liter/dichloroisocoumarin at 20 mg/liter. The solution was stored frozen until required and then diluted 1:100 with the appropriate buffer (all reagents, Boehringer Mannheim).

Antigen Purification. For analytical purposes, a chromosome fraction was prepared according to Huitorel and Kirschner (4) to establish the presence of the 47-kDa protein. To obtain larger quantities of antigen, 50 ml of frozen mitotic or unsynchronized HeLa cells were thawed in 100 ml of lysis buffer (10 mM Tris, pH 7.5/0.2 mM EDTA/1 mM dithiothreitol/protease inhibitors). Cells were swollen for 20 min on ice, homogenized by 20 strokes in a glass homogenizer, and centrifuged at 70,000 × *g* for 60 min. The pellet was preextracted sequentially with lysis buffer containing 80 mM NaCl and with 7.5 mM chloroquine diphosphate (Serva) in lysis buffer. After each extraction step the homogenate was centrifuged for 30 min at 70,000 × *g*. Elution of the 47-kDa antigen was achieved by resuspending the pellet in lysis buffer containing 25 mM chloroquine (21). Alternatively, freshly prepared HeLa cell nuclei (22) from unsynchronized cells were extracted in the same manner but were centrifuged for only 10 min at 2000 × *g* after each step. The cell nuclei retained their shape during the entire extraction procedure.

Ammonium sulfate up to 40% saturation was added to the 25 mM chloroquine extract. After centrifugation for 20 min at 25,000 × *g*, the pellet was discarded, and the 47-kDa antigen was precipitated from the supernatant by 65% saturation with ammonium sulfate. The precipitate was collected by centrifugation and stored at −20°C. The precipitate was then redissolved in 50 mM sodium phosphate, pH 6.8, and applied to a cation-exchange column (Mono S, HR 5/5, Pharmacia). After being washed with starting buffer (50 mM NaCl/50 mM sodium phosphate, pH 6.8), bound proteins were eluted with a linear gradient of NaCl from 0.05 to 1 M (1 ml/min) in this buffer. Fractions containing the antigen were identified by electrophoresis and immunoblotting. These fractions were diluted with two volumes of 10 mM Tris, pH 7.6, loaded on a Mono Q column (HR 5/5, Pharmacia), and eluted with a NaCl gradient (0.1–1 M in 10 mM Tris, pH 7.6, at 1 ml/min).

A 100-μl aliquot of the Mono Q fraction containing the 47-kDa antigen was applied to a Superose 12 HR 10/30 column (Pharmacia) and chromatographed in 300 mM NaCl/10 mM Tris, pH 7.6 at 0.2 ml/min. For separation of the 47-kDa and 25-kDa polypeptides, the antigen fraction was lyophilized, and proteins were redissolved in 100 μl of 6 M guanidinium chloride.

Sequence Determination. The purified antigen complex was reduced in 6 M guanidinium chloride/1 M Tris, pH 8.6/120 mM mercaptoethanol/2.7 mM EDTA for 16 hr at room temperature. Then 1.2 M iodoacetate in H_2O was added to a final concentration of 120 mM. Alkylation was terminated after 15 min by addition of 2 μl of 2-mercaptoethanol, and the sample was chromatographed immediately on Superose 12 HR 10/30 in 6 M guanidium chloride/50 mM Tris, pH 8.6, at 0.2 ml/min. The separated proteins were precipitated with methanol/chloroform, 4:1 (vol/vol), and aliquots were cleaved with endoproteinase N-Asp (Boehringer Mannheim) for 14 hr at 37°C in 0.1 M ammonium bicarbonate, pH 7.5, in 2 M deionized urea or with CNBr/70% formic acid, respectively. The digest was lyophilized and fractionated on a reversed-phase column (ODP50, Asahipak, 4.6 × 150 mm) by using a gradient of acetonitrile in 50 mM ammonium bicarbonate at a flow rate of 1 ml/min. Peptides were sequenced on an Applied Biosystems A470 gas-phase sequencer, and the resulting phenylthiohydantoins were identified and quantitated on-line.

RESULTS AND DISCUSSION

Immunological Characterization of the Autoantigen. Forty four human autoimmune sera recognizing kinetochores in indirect immunofluorescence studies were tested on blots with HeLa nuclear proteins to identify their respective antigens. Of these sera, 11 recognized a 47-kDa major antigen. In addition, all of them stained a protein band of 18 kDa. One serum with a titer of 2×10^4 in immunofluorescence studies (2804) was used to monitor purification of the 47-kDa antigen. In HeLa cells, centromeres were stained only as dot-like structures; the large chromosomes of the Indian muntjak, however, show a rod-shaped fluorescence along the lateral surface corresponding to the kinetochore region (Fig. 1). In immunoblots with HeLa nuclear proteins, this serum recognized three antigens (Fig. 2). The 47-kDa and the 18-kDa bands were heavily stained; in addition, a faint band at 23 kDa was recognized. To correlate immunofluorescence in cells with the antigen-banding pattern on immunoblots, antibodies were eluted from antigenic bands of a preparative blot. As control, antibody was eluted from a nonantigenic protein band at ≈150 kDa. The affinity-purified antibodies were tested in immunofluorescence studies on human HeLa cells and on blots with HeLa total proteins. Antibodies eluted from the 18-kDa band stained this same band and, in addition, stained the bands at 23 and 47 kDa, indicating a common structural motif. Similarly, the 18-kDa band was stained with

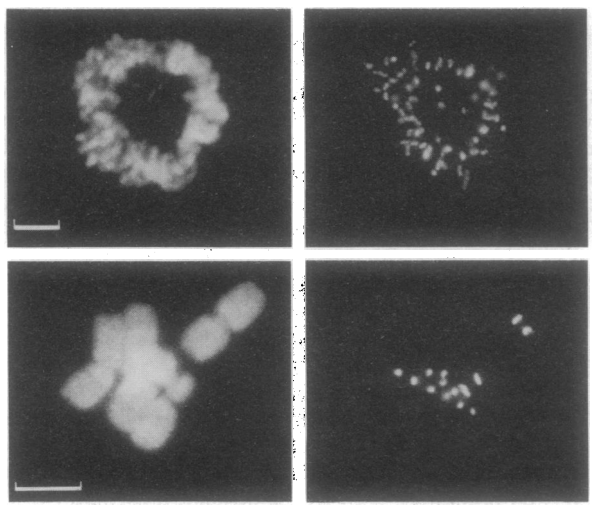

FIG. 1. DNA and antikinetochore immunofluorescence staining of HeLa and muntjak mitotic cells. (*Left*) DNA stained with Hoechst 33342. (*Right*) Same view fields stained with autoimmune serum and fluorescein isothiocyanate-conjugated antihuman serum. (*Upper*) HeLa cell. (*Lower*) Muntjak cell. (Bar = 5 μm.)

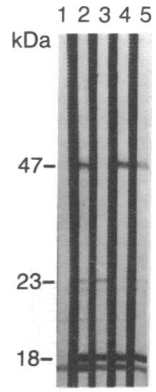

FIG. 2. Crossreaction of antigenic bands stained on immunoblots with affinity-purified antibodies. Autoimmune serum was adsorbed to electrophoretically separated HeLa proteins blotted onto nitrocellulose. Antibodies were eluted from a nonantigenic protein band at 150 kDa (lane 1), the 18-kDa (lane 2), 23-kDa (lane 3), and 47-kDa (lane 4) antigen and used to restain blots. Control staining with complete antiserum is shown in lane 5. The nonspecific band at the bottom of each lane results from core histones.

antibodies eluted from the 23- and 47-kDa antigen bands. The 23-kDa and 47-kDa antigens did not crossreact. All three affinity-purified antibodies recognized the kinetochore structure on HeLa cells, in contrast to the antibody eluted from the control band. Because of the crossreaction of the 47- and 18-kDa antigens, it was not possible to obtain an antibody specific for either protein. Hence, localization of the 47-kDa protein at the kinetochores by immunofluorescence remains elusive.

Antigen Purification. HeLa cells arrested in mitosis, unsynchronized cells, and HeLa nuclei prepared from logarithmically growing cells were used as antigen sources. After disruption of the cells, the high-speed pellet or the purified nuclei were preextracted with 80 mM NaCl/7.5 mM chloroquine. The 47-kDa antigen was released from DNA by intercalation with 25 mM chloroquine (Fig. 3). When nuclei were extracted, they remained morphologically intact. Alternatively, the antigen could be extracted with lower selectivity by using 400 mM sodium chloride in the buffer.

After precipitation with 65% ammonium sulfate, the antigen fraction was chromatographed on a Mono S column (Fig.

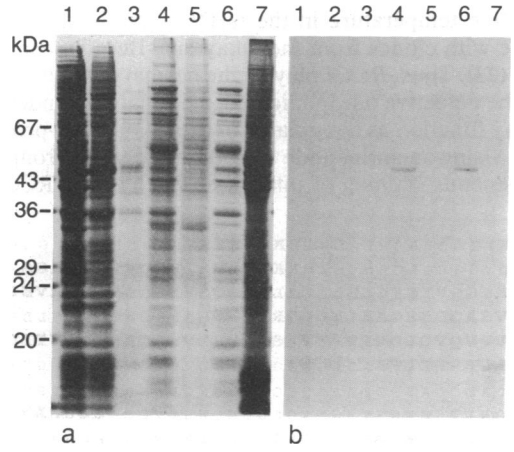

FIG. 3. Elution of the antigen from unsynchronized HeLa cells. Left lanes, staining with Ponceau S. Right lanes, staining with antibody. Lanes: 1, proteins soluble in 10 mM Tris pH 7.5; 2, extraction with the same buffer containing 80 mM NaCl; 3, extraction with the same buffer containing 7.5 mM chloroquine; 4, extraction with the same buffer containing 25 mM chloroquine; 5, precipitation of fraction 4 with 40% (wt/vol) ammonium sulfate; 6, precipitation of fraction 4 with 65% ammonium sulfate; 7, insoluble pellet.

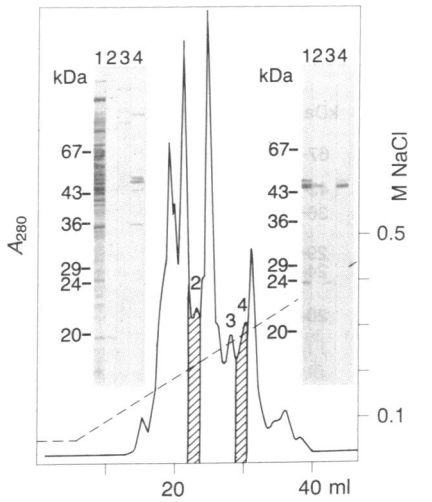

FIG. 4. Chromatography of the 65% ammonium sulfate fraction on a Mono S column. Hatched fractions contain the 47-kDa antigen. (*Left inset*) Staining of immunoblotted ammonium sulfate precipitated sample (lanes 1) and fractions 2, 3, and 4 (respective lanes) with Ponceau S. (*Right inset*) Same blots stained with antiserum.

4). With material from unsynchronized cells, the 47-kDa antigen was eluted in two peaks. Fraction 2 contained only one antigenic band of 47 kDa, whereas in fraction 4 an additional faint band at 50 kDa was stained. The 23-kDa antigen was eluted in fraction 3 in much smaller amounts. The two fractions containing the 47-kDa antigen were chromatographed on a Mono Q column in separate runs. Fig. 5 represents rechromatography of fraction 2 in Fig. 4. In addition to the antigen, there remained only one nonantigenic component; it had a molecular mass of 25 kDa. Nearly the same result was obtained with Mono S fraction 4, where, however, the faint 50-kDa antigen band remained in the 47-kDa antigen fraction. When the material was from highly synchronized (>92%) mitotic cells, the antigen eluted from the Mono S column almost exclusively in fraction 2.

Complex Formation with a 25-kDa Protein. Gel chromatography of the antigen fraction in Fig. 5 was performed in Tris buffer/sodium chloride at concentrations corresponding to the elution point in Mono Q separation. Under these nondenaturating conditions, both proteins were eluted in one peak at 68 kDa, whereas ovalbumin and chymotrypsinogen A from

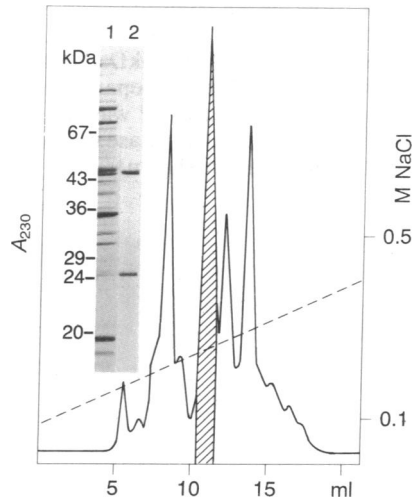

FIG. 5. Chromatography of fraction 2 from a Mono S column on a Mono Q column. Hatched fraction contains the 47-kDa antigen. (*Inset*) Coomassie blue staining after gel electrophoresis. Lanes: 1, total sample applied to column; 2, hatched antigen fraction.

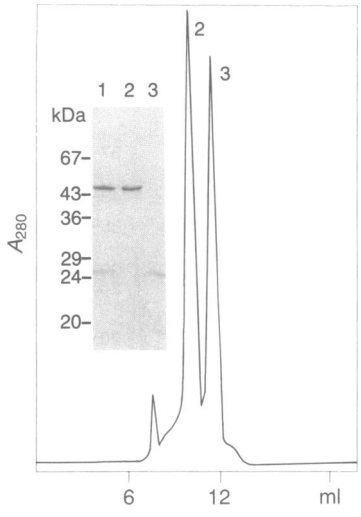

FIG. 6. Gel filtration of the 68-kDa complex in 6 M guanidinium chloride on Superose 12. (*Inset*) Staining of sample applied (lane 1) and of fractions 2 (lane 2) and 3 (lane 3) with Coomassie blue.

a marker mixture were well separated. The two components could be separated by gel chromatography in 6 M guanidinium chloride (Fig. 6). Determination of the protein content of the two peaks indicated that the 47-kDa antigen formed a 1:1 noncovalent complex with the 25-kDa component. The faint 50-kDa band remained associated with the 47-kDa protein and possibly represents a modified form of the latter. We calculate from phenylthiohydantoin yields in sequence determination that ≈300 pmol of each subunit was isolated from 50 ml of cells.

Isoforms of the Antigen: Indications of Posttranslational Modification. From the crossreactions in Fig. 2, we infer that similar epitopes are present on two polypeptides each. This result may indicate related proteins or/and a common posttranslational modification. Several discrete antigenic spots arise in two-dimensional gel electrophoresis. In addition to the two spots in material from mitotic cells (Fig. 7*a*), the fraction eluting later from Mono S separation (Fig. 7*b*), present only in material from unsynchronized cells, reveals four additional spots of a more basic pI. This result suggests cell-cycle-dependent changes of modifications. Furthermore, the N terminus of the protein was devoid of a free α-amino group upon sequencing, and peptides with identical sequence eluted in two or three peaks from reversed-phase columns (Fig. 8). In addition, a faint antigenic band of 50 kDa is consistently associated with the 47-kDa protein in antigen peak 4 eluting later from the Mono S separation. Neither form appears phosphorylated because ^{32}P was not incorporated, and treatment with alkaline phosphatase did not shift pattern or pI of the isoforms (data not shown).

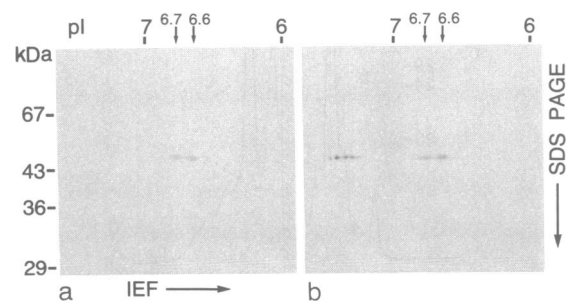

FIG. 7. Two-dimensional gel electrophoresis and immunoblots of isoforms of the 47-kDa antigen after chromatography on a Mono S column (Fig. 4). (*a*) Fraction 2. (*b*) Fraction 4. IEF, isoelectric focusing.

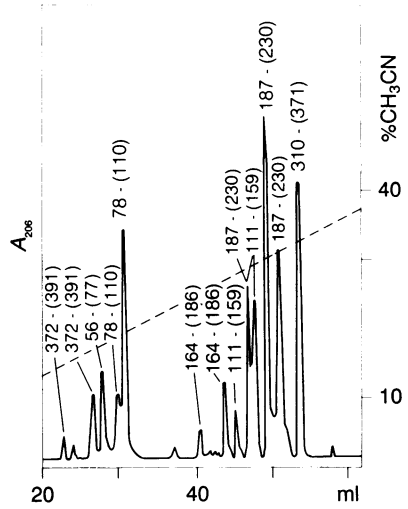

FIG. 8. Fractionation of CNBr-derived fragments of the 47-kDa antigen on a reversed-phase column in 0.1% trifluoroacetic acid with a linear gradient of acetonitrile. Numbers above each peak indicate positions of the respective fragments in the sequence of RCC1 (see Fig. 9).

Sequence Homology of the 47-kDa Protein with RCC1. For sequence determination, the proteins were reduced and alkylated before gel chromatography. The sequence of the 25-kDa protein will be described in a separate communication. This sequence is not homologous to any structure in the public data banks. Neither protein is homologous to the 80-kDa centromere protein B (19). Sequencing of the intact 47-kDa antigen revealed a blocked N terminus in contrast to a control protein in a neighboring fraction. Aliquots of the protein were cleaved with N-Asp protease and CNBr, respectively, the fragments were fractionated on a reversed-phase column (see Fig. 8 for CNBr fragments), and the peptides were sequenced. Two hundred and five residues of the 47-kDa antigen have been determined on the protein level. A search in data banks revealed these amino acid sequences to fit without any exchange into an open reading frame for RCC1 protein (Fig. 9). This structure comprises seven homologous domains of roughly 60 amino acids.

The *RCC1* gene was identified by complementing temperature-sensitive premature chromosome condensation at nonpermissive temperature in the tsBN2 baby hamster kidney cell line with clones from the Okayama–Berg human cDNA library (23). Thus, *RCC1* may be the normal gene corresponding to the defective one implicated in premature condensation and may function as a regulator of the maturation-promoting factor. Using an antipeptide antibody, the same group found homogeneous staining of interphase nuclei. The RCC1 pro-

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MSPKRIAKRRSPPADAIPKSKKVKVSHRSHSTEPGLVLTL
GQGDVGQLGLGENVMERKKPALVSIPEDVVQAEAGGMHTV
CLSKSGQVYSFGCNDEGALGRDTSVEGSEMVPGKVELQEK
VVQVSAGDSHTAALTDDGRVFLWGSFRDNNGVIGLLEPMK
KSMVPVQVQLDVPVVKVASGNDHLVMLTADGDLYTLGCGE
QGQLGRVPELFANRGGRQGLERLLVPKCVMLKSRGSRGHV
RFQDAFCGAYFTFAISHEGHVYGFGLSNYHQLGTPGTESC
FIPQNLTSFKNSTKSWVGFSGGQHHTVCMDSEGKAYSLGR
AEYGRLGLGEGAEEKSIPTLISRLPAVSSVACGASVGYAV
TKDGRVFAWGMGTNYQLGTGQDEDAWSPVEMMGKQLENRV
VLSVSSGGQHTVLLVKDKEQS
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FIG. 9. Amino acid sequence (in one-letter code) of the translated reading frame of *RCC1*. Amino acid sequences derived from the 47-kDa antigen are in boldface type. Single roman letters within boldface sequences indicate positions not identified by protein sequencing. All identified residues correspond to the predicted sequence of RCC1.

tein could be released from nuclei by DNase I or 0.3 M NaCl treatment and from DNA-cellulose columns by elution with the same salt concentration (24).

So far, we cannot claim identity of our antigen with RCC1 protein. Amino acid sequence data, molecular mass, nuclear localization, binding to DNA, and elution from a DNA-cellulose column with 350 mM NaCl (data not shown) indicate a very close relationship, whereas autoimmune antibody affinity-purified from our protein stains kinetochores with very little background in interphase cells. The amount of complex does not vary significantly throughout the cell cycle (data not shown), and we believe the 47- and 25-kDa polypeptides act as partners rather than antagonists. If, indeed, our antigen corresponds to an inhibitor of premature chromosome condensation, it may be regulated by as-yet-unidentified cell-cycle-dependent posttranslational modifications.

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