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CHROMOSOMA


# Centromere proteins

## I. Mitosis specific centromere antigen recognized by anti-centromere autoantibodies

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**Abstract.** Human anti-centromere sera from scleroderma patients were used to detect centromere antigens of mouse fibroblast cells. An $M_r = 59000$ centromere protein was localized exclusively on mitotic chromosomes. The association of this protein with the mitotic chromosomes proved to be DNase I sensitive. In interphase nuclei, this centromere antigen was not detectable by immunoblot techniques. The results suggest that the $M_r = 59000$ mitosis specific protein may be necessary for the structural stability of kinetochores during mitosis.

### Introduction

In recent years, the use of human autoantibodies reacting with centromere antigens (Moroi et al. 1980) has permitted the study of the constituent proteins of mammalian centromeres. A number of antigenic polypeptides have been identified and localized in the centromeric region of chromosomes and in interphase nuclei of mammals (Balczon and Brinkley 1986; Cox et al. 1983; Earnshaw and Rothfield 1985). The existence of antigens recognized by anti-centromere (AC) sera in the nuclei of frog, *Drosophila,* yeast, and *Tetrahymena* has also been reported (Cox and Olmsted 1984). Recently, cloned cDNA encoding an $M_r = 80000$ protein, one of the major centromere antigens of human chromosomes, has been isolated and characterized (Earnshaw et al. 1987).

Our knowledge of the function of centromere proteins recognized by human AC sera is limited. On the basis of biochemical studies the $M_r = 17000$ human centromere protein has been suggested to function as a centromere specific core histone (Palmer et al. 1987).

In this study, we analyzed the centromere antigens of highly purified chromosomes and interphase nuclei of a mouse fibroblast cell line by immunological and biochemical methods. We were able to distinguish "mitotic centromeres" from "interphase centromeres" by the identification of a protein localized exclusively on mitotic chromosomes. Results of preliminary biochemical studies of this mitosis specific centromere protein suggest that it may play a role in the structural stability of kinetochores during mitosis.

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### Materials and methods

*Cells.* Mouse fibroblast cells ($LMTK^-$) were cultured in Nutrient Mixture F-12 medium (Gibco, Scotland) supplemented with 10% fetal calf serum (Human, Hungary).

*Antisera.* Two human AC autoantisera (LU 851, and RJ 8513) were obtained from the Department of Dermatology, Karolinska Hospital, Stockholm, Sweden. Both sera were obtained from patients suffering from a form of progressive systemic sclerosis (PSS or scleroderma) known as CREST. Amongst the well known human centromere antigens (Earnshaw and Rothfield 1985) AC serum LU 851 recognizes the $M_r = 17000$ (CENP-A), 80000 (CENP-B), and 140000 (CENP-C) proteins (Hadlaczky et al. 1986). Serum RJ 8513 recognizes the $M_r = 17000$ and 80000 antigens (unpublished). AC serum D 460 was kindly provided by Dr. J. Schlammadinger, Medical School, Debrecen, Hungary. Serum MP 858 from a healthy donor served as control.

*Isolation of chromosomes and nuclei.* Isolation of chromosomes and nuclei of mouse fibroblast cells was carried out as described previously (Hadlaczky et al. 1981) with the modification that a glycine-hexylene glycol isolation buffer was used (Hadlaczky et al. 1982). Alternatively, chromosomes and nuclei were isolated by the procedure of Gooderham and Jeppesen (1983) utilizing quasi-physiological conditions.

*Indirect immunofluorescence.* For indirect immunofluorescence studies colchicine-blocked mouse cells were used. After 15 min hypotonic treatment (Stenman et al. 1975) cells were flattened by cytocentrifugation, fixed in methanol/acetone 1/1 mixture at −20° C for 5 min then immunostained with AC serum at a 1/1000 dilution as described earlier (Hadlaczky et al. 1986).

*Gel electrophoresis and immunoblotting.* Proteins were separated on 10 × 8 cm, 5%–20% gradient SDS-polyacrylamide gels (Laemmli 1970). Whole cells, isolated nuclei, and chromosomes were dissolved directly in electrophoresis sample buffer, whereas cytoplasmic and extracted proteins were precipitated with 20% trichloroacetic acid, washed with acetone and air dried prior to dissolving.

For immunoblotting SDS-polyacrylamide gel separated proteins were transferred to nitrocellulose filters (Schleicher

and Schuell, BA 83, FRG) according to Towbin et al. (1979) using an imidazole glycyl-glycine buffer (Hadlaczky et al. 1986). Prior to antibody treatment, filters were blocked with phosphate buffered saline containing 1% bovine serum albumin (Sigma, USA) 20% fetal calf serum and 0.05% Tween-20 (BioRad, USA) at 4° C overnight. Binding of human autoantibodies diluted to 1/100-200 in blocking solution or phosphate buffered saline containing 0.05% Tween-20 (Tween-PBS) was detected by peroxidase-conjugated rabbit immunoglobulins to human IgA, IgG, IgM, kappa, lambda (Dakopatts, Denmark) diluted to 1/200 in Tween-PBS or in blocking solution. The enzyme reaction was visualized by the use of BioRad HRP Color Development Reagent.

To estimate the molecular weight of proteins according to their electrophoretic mobility 17 cm long, 5%–20% gradient gels with BioRad LMW and Pharmacia HMW kits (Pharmacia, Sweden) were used. In some cases additional molecular weight markers were used: $\beta$-galactosidase, 130 kDa (Sigma, USA), and cytochrome *c*, 12.4 kDa (Sigma, USA).

*Immunoprecipitation.* Immunoprecipitation using Protein A-Sepharose CL4B (Pharmacia, Sweden) was performed according to Matter et al. (1982).

*Antigen affinity purification of antibodies.* Affinity purification of AC autoantibodies from mouse centromeric antigens immobilized on nitrocellulose filters was carried out according to Olmsted (1981), with the modification that the eluted antibody fractions were neutralized with 1 M Tris buffer, pH 8.5. For indirect immunofluorescence and reblotting, undiluted affinity purified antibodies were used.

*Nuclease treatment.* Suspensions of isolated mouse chromosomes and nuclei were treated with 50–100 µg/ml DNase I (Worthington, USA) at 37° C for 1 h. RNase treatment was carried out with 100 µg/ml RNase A (Sigma, USA) at 37° C for 1 h. Nuclease-free buffer (10 mM Tris, 100 mM NaCl, 2.5 mM $MgCl_2$, pH 7.5) treatment under the same conditions served as control.

For indirect immunofluorescence studies samples were treated with 1–10 µg/ml DNase I (100–200 µl/slide) at 37° C for 45 min.

## Results

Mouse fibroblast cells ($LMTK^-$) probed with human AC serum (LU 851) showed a typical centromere staining pattern by indirect immunofluorescence. Positive immunostaining was detectable at the centromeres of the mitotic chromosomes and also in the interphase nuclei as discrete spots (Fig. 1). In some cases weak immunostaining of centrioles was also detected. Apart from centromere staining, no other immunopositivity was detected on the chromosomes or in the interphase nuclei.

To identify the exact polypeptide targets of antibodies immunoblotting analysis of different protein fractions of mouse cells (Fig. 2a) was performed. Figure 2b shows the immunoperoxidase staining pattern of proteins from whole cells (lane 1), the cytoplasm of interphase (lane 2) and mitotic (lane 3) cells, isolated nuclei (lane 4), and isolated mitotic chromosomes (lane 5). In whole cells, serum LU 851

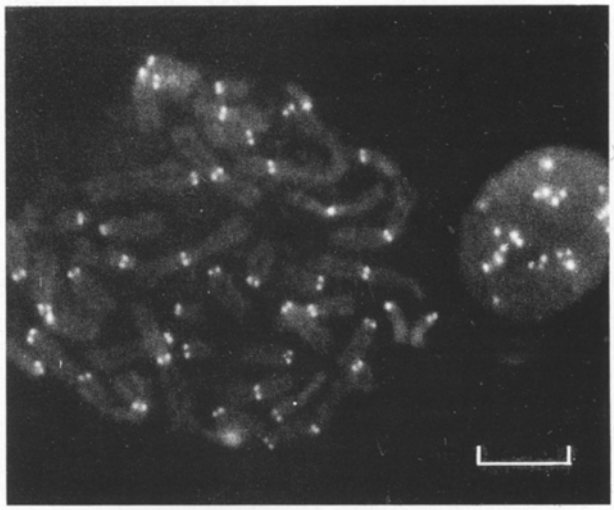

**Fig. 1.** Indirect immunofluorescence staining pattern of chromosomes and nucleus of mouse fibroblast cells with anti-centromere (AC) serum LU 851 at 1/1000 dilution. Bar represents 10 µm

recognized a set of antigens with apparent $M_r$s of 17000, 23000, 30/31000 (doublet), 46/47000 (doublet), 145000, and 160000. The antigen patterns in cytoplasmic fractions of mitotic and interphase cells were identical (compare lanes 3 and 2). Comparing these with the pattern in whole cells, the relative amount of the $M_r = 30/31000$ antigens was increased in the cytoplasmic fractions. The antigen pattern of the interphase nuclei was similar to that of whole cells but several weak antigens were found in the $M_r = 72$–94000 molecular weight range and no $M_r = 30/31000$ cytoplasmic antigens were detected (Fig. 2b, lane 4). In the chromosome fraction, qualitative differences in the antigenic polypeptide pattern were detected. In addition to the nuclear antigens, an extra antigenic polypeptide was found in isolated mitotic chromosomes with an apparent $M_r$ of 59000 (Fig. 2b, lane 5; Fig. 2c, lane 1).

The stabilizing agent hexylene glycol used in our chromosome isolation medium may cause contamination of chromosomes and nuclei by non-nuclear proteins. To rule out the possibility of a non-nuclear origin of the $M_r = 59000$ antigen, we compared the antigen pattern of chromosomes and nuclei isolated by a procedure utilizing quasi-physiological conditions (Gooderham and Jeppesen 1983) and the hexylene glycol procedure. We found no qualitative differences in the immunostaining pattern of major antigens (not shown).

Further characterization of antigenic proteins and antibodies specific to them was carried out by immunoblotting with antigen affinity purified antibodies of AC serum LU 851. Antibody fractions eluted from the excised antigen-containing regions of an immunoblot were used for reblotting on chromosomal proteins. Figure 2c shows the results of a reblotting experiment. All but one of the affinity purified antibodies (Fig. 2c, lane 8) reacted with the "target" antigens and to a certain extent crossreactivity of affinity purified antibodies could also be detected. Because of the different reactivities of the affinity purified antibodies to the individual antigens (compare Fig. 2c, lanes 4 and 5, and lanes 6 and 7), crossreactions as the result of crosscontamination of different antibodies (e.g. aggregation of different

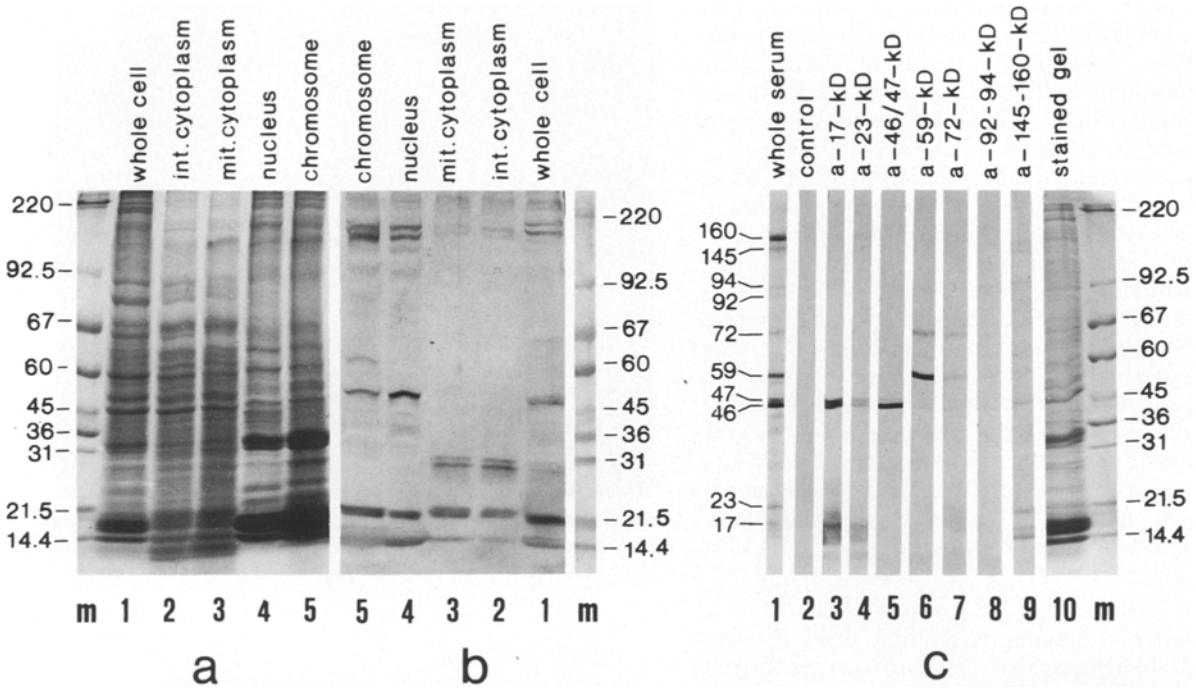


**Fig. 2a–c.** Immunoblotting analysis of SDS-polyacrylamide gel fractionated mouse proteins with AC serum LU 851 and with antigen affinity purified antibodies. **a** Coomassie Blue stained gel. *Lane 1* proteins of whole cells; *lane 2* cytoplasm of interphase cells; *lane 3* cytoplasm of mitotic cells; *lane 4* isolated interphase nuclei; *lane 5* isolated mitotic chromosomes; *lane m* molecular weight markers. **b** Immunoperoxidase staining of antigens recognized by AC serum in different fractions of mouse cells. *Lanes 1–5* as in **a**; *lane m* Amido Black stained molecular weight markers transferred to nitrocellulose filter. **c** Immunoperoxidase staining of chromosomal proteins with antigen affinity purified antibody fractions of AC serum LU 851. *Lane 1* antigen pattern obtained by whole serum at 1/200 dilution; *lane 2* control, eluted from the excised $M_r = 25$–$28000$ antigen-free region. Antigens recognized by: *lane 3* the anti-17000 $M_r$ antibody; *lane 4* anti-23000 $M_r$ antibody; *lane 5* anti-46/47000 $M_r$ antibody; *lane 6* anti-59000 $M_r$ antibody; *lane 7* anti-72000 $M_r$ antibody; *lane 8* anti-92–94000 $M_r$ antibody, *lane 9* anti-145–160000 antibody. *Lane 10* part of Coomassie Blue stained gel which was used for blotting, showing the protein pattern of isolated chromosomes. *Lane m* molecular weight markers

immunoglobulins) seem to be unlikely. Crossreactivity between affinity purified human AC antibodies (Earnshaw and Rothfield 1985) led to the conclusion that centromere proteins are immunologically related sharing structural determinants (Earnshaw et al. 1987). This seems to be true in our case for mouse centromere antigens. From reblotting experiments it is clear that the LU 851 serum contains at least four different antibodies specific to: (1) $M_r = 17000$, 23000, 46/47000, 145000, and 160000 proteins (Fig. 2c, lanes 3 and 4); (2) an $M_r = 46000$ protein (Fig. 2c, lane 5); (3) an $M_r = 59000$ and a minor $M_r = 72000$ protein (Fig. 2c, lanes 6 and 7); (4) $M_r = 160000$, 145000, 94000, 46000, 23000, and 17000 proteins (Fig. 2c, lane 9). It is also evident that these antibodies recognize different epitopes which are present: (a) predominantly on the $M_r = 46000$ protein (Fig. 2c, lane 5); (b) approximately in equal amounts on the $M_r = 46000$ and 47000 proteins and shared by the $M_r = 17000$, 23000, 145000, and 160000 antigens (Fig. 2c, lanes 3 and 4); (c) on the $M_r = 59000$, and 72000 antigens (Fig. 2c; lanes 6 and 7); (d) on the $M_r = 145000$, 160000, 94000, 46000, 23000, and 17000 antigens (Fig. 2c, lane 9).

Eluate from the excised $M_r = 25$–$28000$ region which is free from antigen served as a negative control. No immunoreactive material was obtained from this region (Fig. 2c, lane 2). Presumably due to the low concentration of antibodies recovered from the $M_r = 92$–$94000$ region, results obtained with this fraction (Fig. 2c, lane 8) were inconclusive in repeated experiments. However, the anti-92–94000 $M_r$ fraction was successfully used in indirect immunofluorescence experiments (see below).

The results of the reblotting experiments with antigen affinity purified antibodies indicate that the $M_r = 59000$ mitosis specific polypeptide is not related to the major antigenic polypeptides. This is further supported by the fact that an AC serum, RJ 8513, which recognized the $M_r = 46/47000$ antigens did not react with the $M_r = 59000$ polypeptide (Fig. 3c). Another AC serum, D 460, of which the $M_r = 72000$ polypeptide is a strong antigen (Fig. 3d) did not react with the $M_r = 59000$ mitosis specific antigen. On the basis of these results we considered the $M_r = 59000$ antigen to be a distinct chromosomal protein.

To determine whether the antigenic proteins are components of the centromere or not, affinity eluted antibodies were used to localize the antigens in mitotic and interphase cells by indirect immunofluorescence. Except for the control, all of the affinity eluted antibody fractions stained both mitotic and interphase centromeres although with different intensities. The anti-59000 $M_r$ antibody stained exclusively the centromeric region of chromosomes and no sign of noncentromeric staining was detectable (Fig. 4a). Considering the extreme sensitivity of the immunofluores-

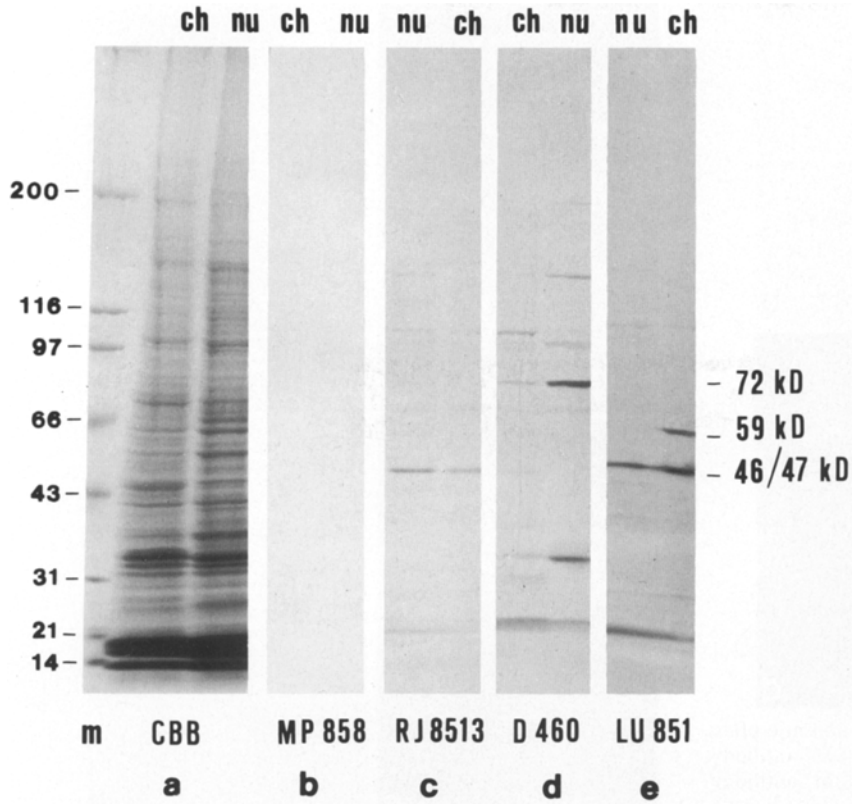


**Fig. 3a–e.** Immunostaining of chromosomal and nuclear antigens of mouse with AC sera. **a** Coomassie Blue stained gel. Immunostaining with: **b** control serum MP 858 from a healthy donor containing no AC antibodies; **c** AC serum RJ 8513; **d** AC serum D 460; **e** AC serum LU 851. *Lanes ch* proteins of isolated chromosomes; *lanes nu* proteins of isolated nuclei. *Lane m* molecular weight markers

cence technique, if the localization of the $M_r = 59000$ antigen is noncentromeric such a strong antigen (Fig. 2c, lane 6) would result in positive noncentromeric immunostaining even if the antigen were homogeneously distributed. The anti-59000 $M_r$ antibody showed significantly stronger centromere staining on chromosomes than in the interphase nucleus (Fig. 4a). A reverse situation was found with the anti-92–94000 and anti-46000 $M_r$ antibodies which stained the interphase centromeres more intensely (Fig. 4c and d). The weak staining of interphase centromeres with the anti-59000 $M_r$ antibody can be attributed to the reactivity of this antibody with the $M_r = 72000$ antigen (Fig. 2c, lane 6) present in the interphase nucleus (Figs. 2b, lane 4; 3d, lane nu). The difference in intensity of staining of the interphase and mitotic centromeres with the anti-92–94000 and anti-46000 $M_r$ antibodies can be explained by the quantitative differences in the amounts of antigens in interphase and mitotic centromeres (Fig. 2a, b, lanes 4 and 5).

In summary, the data from the experiments described so far demonstrate that the antigenic polypeptides recognized by AC serum LU 851 are centromeric proteins including an $M_r = 59000$ protein which is detectable only in the mitotic centromere.

To determine whether the $M_r = 59000$ centromere protein interacts directly with chromosomal DNA, isolated chromosomes were exposed to extensive DNase I treatment and the solubilized proteins analyzed by immunoblotting. The $M_r = 59000$ centromere antigen was found to be most sensitive to DNase I treatment. Treatment with 50 µg/ml DNase I for 1 h extracted more than 50% of this protein, whereas only a small amount of the $M_r = 46000$ antigen was released (Fig. 5a, lanes 3 and 4). Treatment with 100 µg/ml DNase I with resulted in complete extraction of the $M_r = 59000$ antigen (not shown). Extensive RNase A (Fig. 5a, lanes 5 and 6) or nuclease-free buffer treatment (not shown) did not result in the release of detectable centromere antigens into the supernatant.

The consistent extraction of a small quantity of the $M_r = 46000$ antigen together with the $M_r = 59000$ protein could be explained by their partial association. To test this assumption, isolated nuclei which contain no $M_r = 59000$ antigen were DNase treated. The result did not support the above possibility since about the same proportion of $M_r = 46000$ centromere antigen could be extracted from both interphase nuclei and mitotic chromosomes by-DNase I treatment regardless of the presence or absence of the $M_r = 59000$ antigen (not shown).

Recovery of the DNase I extracted $M_r = 59000$ protein from the supernatant was only possible after low speed (1000 *g*) centrifugation. Higher centrifugal forces (15000 *g*) pelleted the antigen. This suggests that DNase I extracted $M_r = 59000$ protein may be in the form of protein-protein or nucleoprotein complexes. To ascertain whether fast sedimentation of the extracted protein was dependent on DNA fragments associating with the $M_r = 59000$ protein, immunoprecipitation from the supernatant of DNase extracted chromosomes was carried out using immunoglobulins of AC serum LU 851 coupled to Protein A – Sepharose. In fact, we managed to isolate DNA fragments from the immunoprecipitate. DNA fragments were end-labeled with [$\alpha$-$^{32}$P]dATP by Klenow polymerase, previously treated with Proteinase K and RNase A, and run on a 1% agarose gel. On the subsequent autoradiograph, DNA fragments of up to several kilobase pairs were detected (not shown).

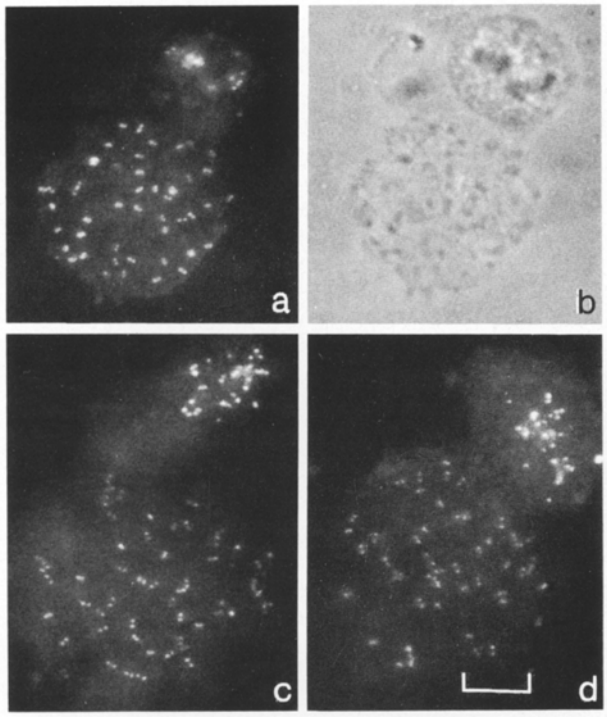


**Fig. 4a–d.** Immunofluorescence patterns of mitotic and interphase cells with affinity purified antibodies. **a** Anti-59000 $M_r$ antibody. **b** Phase contrast micrograph of **a**. **c** Anti-92–94000 $M_r$ antibody. **d** Anti-46/47000 $M_r$ antibody. Bar represents 10 μm

To answer the interesting question of the effect of the removal of the $M_r = 59000$ centromere protein on centromere morphology interphase cells and metaphase chromosome spreads were treated in situ with DNase I for 45 min at 37° C and examined by subsequent indirect immuno-

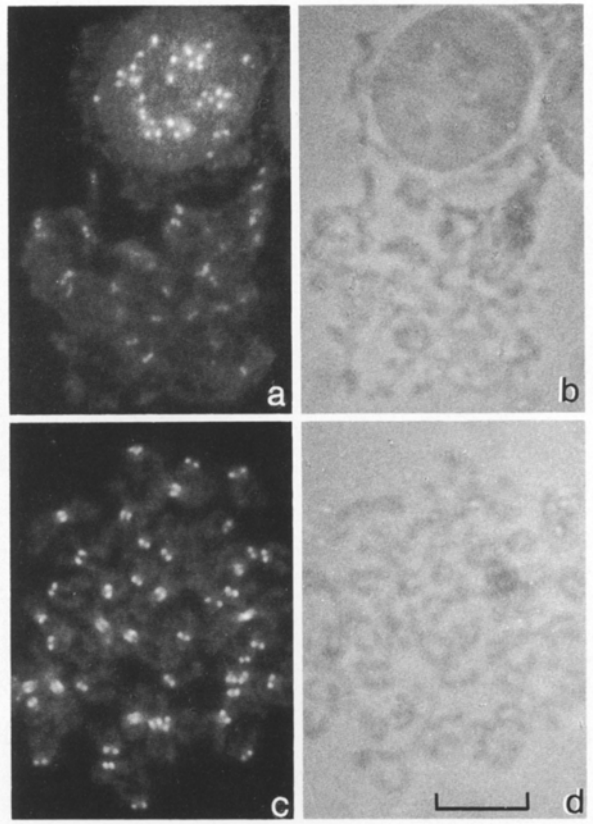


**Fig. 6a–d.** Effect of the removal of $M_r = 59000$ centromere protein on the immunofluorescence patterns of mitotic and interphase mouse cells. **a** Immunofluorescence staining patterns of DNase I treated chromosomes and nucleus with AC serum LU 851. **b** Phase contrast micrograph of **a**. **c** Control, immunofluorescence staining pattern of chromosomes treated with nuclease-free buffer. **d** Phase contrast micrograph of **c**. Bar represents 10 μm

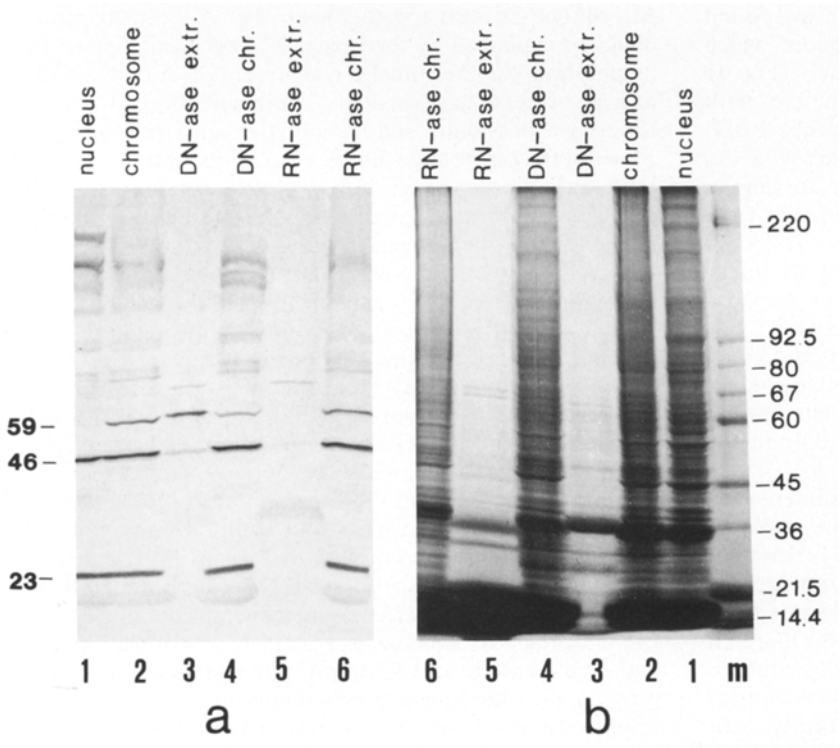


**Fig. 5a, b.** Immunoblotting analysis of nuclease sensitivity of centromere antigens of mouse cells. **a** Immunoblot of mouse proteins reacted with AC serum LU 851 and **b** Coomassie Blue stained gel. Antigen pattern of isolated nuclei, *lane 1;* isolated chromosomes, *lane 2*; DNase I (50 μg/ml) extract of chromosomes, *lane 3*; DNase I treated chromosomes, *lane 4*; RNase (100 μg/ml) extract of chromosomes, *lane 5*; RNase A treated chromosomes, *lane 6*. *Lane m* molecular weight markers

fluorescence. DNase I treatment resulted in a marked reduction in the intensity of centromere staining on chromosomes and a significant reduction in the diameter of the immunopositive area. However, the compact dot-like centromere morphology remained unaffected (Fig. 6a). The staining of interphase centromeres was not affected by treatment with DNase (Fig. 6a; for a comparison see Figs. 1 and 4c, d). Nuclease-free buffer treatment also showed no effect on centromere staining of chromosomes (Fig. 6c).

## Discussion

In this study, we have used human AC sera to identify centromeric proteins of mouse fibroblast cells by indirect immunofluorescence and immunoblotting.

Results with antigen affinity eluted antibodies proved that several strong ($M_r = 17000$, 23000, 46/47000, 59000, and 145/160000) and weak ($M_r = 72000$ and 92/94000) centromeric antigens can be detected in mouse chromosomes by AC serum LU 851. This is in accord with previous results obtained by comparison of human, frog, *Drosophila*, and *Tetrahymena* nuclear proteins (Cox and Olmsted 1984) and of human and hamster centromere antigens (Valdivia and Brinkley 1985).

The presence of shared epitopes on different mouse centromere antigens indicated that some of the proteins were related. It is likely that the centromere proteins identified in this study do not represent all of the existing centromere constituents. The number of different proteins indicates that the centromere is a structure of high complexity. The observation that centromere proteins of mouse chromosomes are recognized by human AC autoantibodies suggests that certain centromere constituents of mammalian chromosomes are evolutionarily conserved. Consequently, centromeres are to some extent conservative structures.

Our results are inconsistent with the previous findings that human AC antibodies fail to give positive centromere reaction on mouse fibroblast cells and mouse tissue sections (Beutner et al. 1985). AC serum LU 851 was successfully been used for detecting centromeres in human (Nyman et al. 1986), Indian muntjac and rat-kangaroo cell lines (Hadlaczky et al. 1986) as well as in different mouse fibroblast (L, A9) and mouse teratocarcinoma (F9, PCC7) cell lines, and in cells of normal mouse tissues (brain, liver, and testis, unpublished). We think that diversity of the AC sera and/or differences in the methods used are the most likely origins of this discrepancy.

We have demonstrated the existence of a mitosis specific centromere antigen in mouse chromosomes. This antigen proved to be a protein with an apparent $M_r$ of 59000. The fact that this protein can be found exclusively in mitotic chromosomes suggests that its presence is necessary for the active, functioning centromere. We have demonstrated that the association of the $M_r = 59000$ mitosis specific centromere protein with the chromosomes is DNase I sensitive and extensive DNase treatment resulted in the extraction of this protein with associated DNA fragments. The indirect immunofluorescence experiments showed that removal of the $M_r = 59000$ protein resulted in a detectable decrease in size of the immunoreactive centromere region. This may indicate a peripheral localization of the antigen. Taking these facts together, we suggest that the $M_r = 59000$ protein may be involved in the physical stability of centromeres in mitotic chromosomes by anchoring the kinetochores to the adjoining chromatin fibers at the perimeter.

An important question that may now be addressed is whether the presence of this mitosis specific protein in the centromere is a unique property of mouse chromosomes or is a more general phenomenon in mammalian chromosomes. Recently, we have also detected an $M_r = 59000$ centromere protein in human chromosomes (Hadlaczky et al. 1986). This human centromere protein proved also to be mitosis specific and its association with the chromosomes was DNase sensitive (in preparation). This raises the possibility that the $M_r = 59000$ protein may be a conserved component of the centromeric regions of mammalian chromosomes.

Efforts to characterize the DNA sequences which possibly bind to the $M_r = 59000$ protein are currently in progress.

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