Monoclonal Antibodies Specific for Tight-binding Human Chromatin Antigens Reveal Structural Rearrangements within the Nucleus during the Cell Cycle

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ABSTRACT The class of nonhistone chromosomal proteins that remains bound to DNA in chromatin in the presence of 2.5 M NaCl-5 M urea has proven refractile to biochemical analysis. In order to study its role in chromatin organization, we have produced monoclonal antibodies that are specific for the HeLa DNA-protein complex that remains after extraction of chromatin with high salt and urea. The antibody-producing clones were identified with an ELISA assay. Of the six clones selected, five were stabilized by limiting dilution. All clones are IgG producers. None cross-react significantly with native DNA, core histones, or the high-mobility group nonhistone proteins. All antibodies are specific for nuclear or juxtanuclear antigens. Indirect immunofluorescence shows that three antibodies, which are nonidentical, stain three different nuclear networks. Available evidence indicates that two of these networks are the nuclear matrix. A fourth antibody reveals structures reminiscent of chromocenters. A fifth antibody, AhNA-1, binds to interphase HeLa chromatin and specifically decorates metaphase chromosomes. AhNA-1 similarly recognizes rat chromosomes. Each of these monoclonal antibodies also reveals a changing pattern of nuclear staining as cells progress through the cell cycle. Presumably, this reflects the rearrangement of the cognate antigens.

The eucaryotic chromosome contains at least twice as much protein by mass as DNA and both are complexed in a structure defined as chromatin. Treatment of either purified nuclei or chromatin by high salt (2.0 M NaCl) removes almost all histones and most nonhistone proteins (1–3). The proteins remaining bound to DNA in chromatin after high ionic strength extraction (2 M NaCl or 2 M NaCl-5M urea) are nonhistones, and they constitute 5–8% of the total chromatin protein. These are termed residual or tight-binding proteins (4–6). The tight-binding, nonhistone chromatin proteins (TBP) from animal cell nuclei are electrophoretically complex (~200 species, Bhorjee, J. S. and L. Kifle, unpublished data), and are distributed nonrandomly along the DNA molecule (reference 5, reviewed in reference 6).

Two functions for these proteins have been suggested, although no direct evidence exists. Based on the DNA sequence-specific association of certain nonhistone proteins with chromatin DNA, a role in specific gene expression for these proteins has been proposed (7–13). It has also been demonstrated that some TBPs bind to androgen-receptor (14) and progesterone-receptor (15) complexes. Thus, at least some gene regulatory molecules in eukaryotes may reside in this group of chromosomal proteins. A second function may be structural. The structural integrity of the nuclear matrix (4, 16) and the chromosome scaffold (17) seems to rely on the presence of such high-ionic strength (2 M NaCl), nonextractable nuclear nonhistone proteins.

Closer examination of these proposals by biochemical analysis of individual tight-binding nonhistone proteins has not been possible for two reasons: (*a*) each is present in small amounts and (*b*) dissociation of the DNA-protein complex requires denaturing solvents that may lead to irreversible loss of functions. Therefore, to avoid these problems and to study the structure and function of HeLa tight binding chromatin proteins, we have prepared monoclonal antibodies against this group of nonhistone proteins. We present here an initial characterization of several HeLa TBP-specific monoclonal antibodies and their antigens. In addition, we use indirect

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immunofluorescence to demonstrate that the spatial distributions of these antigens change dramatically during the cell cycle.

MATERIALS AND METHODS

Cell Growth, Chromatin Isolation, and TBP Fractionation: HeLa cells were grown in suspension cultures in Joklik-modified Eagle's medium (18), and chromatin was purified from isolated nuclei by sedimentation through 60% sucrose as previously described (19). TBPs were fractionated from purified chromatin by treatment with 2.5 M NaCl-5 M urea followed by sedimentation of the TBP-nucleoprotein complex at 408,000 *g* for 24 h as described (5). This TBP-nucleoprotein complex was used as an immunogen. All steps were performed at 4°C and all solutions contained 0.1 mM PMSF as protease inhibitor. Where indicated, cells were synchronized by the double-thymidine block procedure as described previously (20).

Immunological Detection of Proteins by Electrophoretic Blotting: The TBPs were dissociated from the TBP-nucleoprotein complex, as obtained above, by treatment with 2% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol (BME), at room temperature for 30 min with occasional vortexing. The sample was then dialyzed overnight against Tris-NaCl buffer (0.01 M NaCl, 0.0025 M Tris-HCl, pH 7.2) containing 1% SDS-1% BME, and centrifuged at 150,000 *g* for 20 h in Beckman SW50.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) at 20°C, to pellet DNA. Almost all of the protein (>99%) was in the supernatant and DNA (>98%) was in the pellet, as determined by [^{14}C]leucine and [^{3}H]thymidine labeling, respectively. The cytoplasmic proteins were obtained by centrifuging the postnuclear supernatant (19) at 3,000 *g* for 15 min in Sorvall SS-34 rotor at 4°C. The nuclear matrix proteins were obtained by the procedure of Berezney and Coffey (16), except for the pretreatment of isolated nuclei with DNase I (100 μg/l mg DNA) for 30 min at 22°C to avoid gel formation in the high-salt buffer treatment step. Various protein samples (TBPs; nuclear matrix; cytoplasmic) were treated with 1% SDS-1% BME, heated at 90°C for 2 min, and dialyzed against Laemmli sample buffer (21) before electrophoresis.

The electrophoretic transfer and immunological detection of the electroblots was carried out essentially as described by Towbin et al. (22). Briefly, the proteins were first separated in a 11% polyacrylamide SDS-slab gel and transferred electrophoretically to a nitrocellulose membrane sheet using Hoeffer "Transblot" apparatus. The electrophoretic blot membrane strips were incubated with a twofold dilution of the monoclonal culture supernatant, washed, and further incubated with ^{125}I-labeled goat anti-mouse IgG (Amersham Corp., Arlington Heights, IL). The strips were then washed free of the unreacted secondary antibody and exposed to Kodak X-Omat AR film in the presence of an intensifying screen.

Plasmacytoma Culture Conditions: The nonsecreting, 8-azaguanine-resistant mouse plasmacytoma line SP2/0-Ag14 was grown in high glucose Dulbecco's modified Eagle's medium (DME) supplemented with 20% agamma horse serum (KC Biologicals), penicillin, and streptomycin. Cultures were maintained between 10^5 and 7×10^5 cells/ml at 37°C in a humid chamber containing 5% CO_2.

Immunization and Preparation of Hybridomas: BALB/c mice (12–16 wk old) were immunized by intraperitoneal injection of 0.5 ml of immunogen in PBS (100 μg/ml). Animals were boostered at 3-wk intervals by i.p. injection as above. 60–72 h after the sixth injection (into the tail vein), dissociated spleen cells were prepared from an immunized animal and were fused with SP2/0 cells using 50% polyethylene glycol (PEG 1500, Fisher Scientific Co., Pittsburgh, PA) as described by McKearn et al. (23). Treated cells were incubated overnight at 37°C in 5% CO_2 in high glucose DME containing 20% agamma horse serum. Cells were harvested the following day and were resuspended in high glucose DME containing 20% agamma horse serum, 1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, 3×10^{-4} M thymidine, L-arginine (0.116 g/l), L-glutamine (0.216 g/l), folic acid (0.006 g/l), L-asparagine (0.036 g/l), sodium bicarbonate (2.0 g/l), and sodium pyruvate (0.11 g/l). Cells were distributed in 100-μl aliquots into 400 or more microtiter wells and incubated at 37°C in 5% CO_2. 5–6 d later, hybridomas were fed with an additional 100 μl of the above-described medium lacking hypoxanthine, aminopterin, and thymidine. A similar protocol using Freund's adjuvant gave a similar frequency of positive hybridomas.

Screening of Hybridomas and Preparation of Monoclonal Antibodies: Positive hybridomas were detected by an ELISA assay (24). The high salt-urea insoluble DNA-protein complex prepared as described above was diluted to 10–20 μg protein/ml PBS and distributed in 50-μl aliquots into polyvinylchloride microtiter wells. After 2 h at 20°C, each well was emptied, washed four times with PBS, filled with 100 μl of 3% BSA, 0.1% sodium azide in PBS, sealed with tape, and stored at 4°C for up to 2 d before use. Polyvinylchloride microtiter plates coated with immunogen were washed in PBS, washed once for 30 min in 3.0% BSA in PBS, filled with 50 μl of hybridoma culture supernatant, and incubated for 2 h at 20°C. Wells were then washed five times with solution A (PBS + 0.1% Tween-70) and filled with 100 μl of a 1:200 dilution of β-galactosidase-conjugated F(ab')$_2$ sheep or rabbit anti-mouse IgG (H & L) (Bethesda Research Laboratories, Gaithersburg, MD) in solution A. After 2 h at 20°C, each well was washed five times in solution A and filled with 100 μl of 5-bromo-4-chloro-indolyl-β-D-galactoside in PBS (100 μg/ml). After 30–60 min at 20°C, a blue color developed in wells that received culture supernatants containing antibody specific for an antigen in the DNA-protein complex. For ELISA assays using PVC plates coated with histones or the high mobility group proteins (HMGs), the procedure described above was followed.

Indirect Immunofluorescence: An aliquot (1 ml) of actively dividing or synchronized HeLa cells was harvested, washed, and suspended at the same concentration in PBS. One or two drops of washed cells were placed on poly-L-lysine-precoated slides, and the cells were fixed in periodate-lysine-paraformaldehyde as described by McLean and Nakane (25). Fixed cells were incubated for 45 min with 100 μl of hybridoma culture supernatant, washed for 45 min in three changes of PBS, and then incubated for 45 min with a 1:50 dilution in PBS of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Tago). After a final 45-min wash in three changes of PBS, the preparations were viewed microscopically and fluorescence was recorded with Kodak Ektachrome 400 film.

RESULTS

Preparation of Immunogen and Generation of Hybridoma Clones

The DNA-protein complex that remains after extraction of HeLa chromatin with 2.5 M NaCl-5 M urea was used as an immunogen to raise monoclonal antibodies. Some physical and chemical properties of this group of high-affinity DNA-binding nonhistone proteins have been previously described. For example, the TBPs consititue 5–8% of the total chromatin protein, are nonhistones, are not related to the proteins of the heterogeneous ribonucleoprotein particles, and are distributed asymmetrically along the DNA molecule (5).

This nucleoprotein complex is a weak immunogen. In two separate fusions, not more than 2% of the hybridomas secreted antibodies that reacted with the respective antigens as determined by the ELISA assay (24). At least five injections were necessary before a weak positive antibody response could be detected in the immune sera. We have identified six positive clones, and five of these have been stabilized by several subclonings, thus assuring the monoclonal nature of each of the stabilized clones.

Characterization of the Monoclonal Antibodies

We examined five stabilized hybridoma lines for their antigen specificity by using the ELISA assay (see Materials and Methods). All five hybridomas are IgG producers. Table I shows that the antigen-antibody binding reaction is specific for the 2.5 M NaCl-5 M urea stable nucleoprotein complex (TBP). We have numbered the antibody-producing clones with the prefix AhNA for anti-human nuclear antigen. Clones AhNA-4 and AhNA-5 give a strong ELISA reaction and AhNA-2 gives the weakest response. All antibodies are reactive with the native complex of HeLa TBP (column *3*). This reactivity remains even after digestion of the native complex with DNase I (column *9*). HeLa double-stranded DNA does not react with these antibodies. The weak reactions of HeLa single-stranded DNA and HeLa HMGs occur only when these components are present in the assay in amounts that are far greater than are found in the native complex. The weak reaction of some antibodies with preparations of HeLa cytoplasm is expected to result from dispersal of some nuclear antigens into the cytoplasm arising from a minimum number

TABLE I

ELISA Assay of Subcellular Fractions

Clone	Class*	Antigen source: HeLa TBP (native complex)	HeLa dsDNA	HeLa ssDNA	HeLa HMGs	HeLa CP	HeLa NM	DNase I sensitivity of the TBP complex‡
AhNA1	IgG	3+	–	±	–	±	–	–
AhNA2	IgG	3+	–	±	–	–	–	–
AhNA3	IgG	3+	–	±	–	–	2+	–
AhNA4	IgG	4+	–	±	±	±	2+	–
AhNA5	IgG	4+	–	±	±	±	±	–
AhNA6	NT	3+	NT	NT	NT	NT	NT	NT

* Determined by Ouchterlony assay.
+, Indicates the rate of reaction; for example, 4+ wells positive in 20 min; 3+ in 45 min; 2+ in 90 min. The amount of antigen per well within a column was kept constant. Also, in assays for TBP, HMGs, CP, and NM ~0.75 μg of total protein was added per well.
±, Slightly above background.
NT, Not tested.
–, Background, no color.
‡ The TBP complex was treated with 100 μg pancreatic DNAase I/mg DNA at 37°C for up to 4 h. Controls with DNAase alone used as an antigen showed no cross reaction; and incubation of the TBP-complex antigens at 37°C for 4 h did not diminish the reaction.
HMG, High mobility group nuclear proteins prepared as 5% perchloric acid extracts from which histone H1 has been selectively removed by differential acetone precipitation (43). The presence or absence of H1 in the HMG preparations had no effect on the ELISA reaction.
NM, Nuclear protein maxtrix obtained by published methods (16).
CP, Cytoplasmic proteins.

TABLE II

Species Specificity and the Cellular Distribution of the HeLa DNA-TBP Chromatin Antigen as Determined by Indirect Immunofluorescence

Clone	Cellular localization	Cell type: HeLa	Rat muscle	Distribution Pattern	Pattern frequency*
AhNA1	Nuclear	+	+	Metaphase chromosomes; punctate in interphase cells	>90% punctate (Fig. 1, bottom right); 5–10% chromosomes
AhNA2	Nuclear	+	–	Ranges from one large spot or crescent-shaped to granular (chromocenter?)	60–65% crescent-shaped; 20–30% large spot; 5% weak to no reaction
AhNA3	Nuclear	+	–	Matrixlike pattern	>70% matrixlike; remainder threadlike to weak reaction
AhNA4	Nuclear	+	±	Matrixlike pattern	80% matrixlike; remainder weak to no reaction
AhNA5	Nuclear	+	±	Mostly matrix pattern	70–75% matrixlike (similar to AhNA3)
AhNA6	Nuclear	+	NT	Patchy	80% patchy 20% weak reaction

NT, not tested.
±, faint fluorescence.
* In all cases a minimum of 200 cells were observed.

of mitotic cells in the unsynchronously growing population or synthesis of the antigen in the cytoplasm. The reaction of two of these antibodies with HeLa nuclear matrix proteins is analyzed later in this paper.

Cellular Localization and Distribution Pattern of the Antigens by Indirect Immunofluorescence

Table II lists the immunofluorescent staining properties of antibodies produced by the five stabilized clones and by one clone (AhNA-6) that has not been stabilized. Two mammalian cell lines (HeLa and L8 rat skeletal muscle) were tested by immunofluorescence. Fig. 1 shows the immunofluorescent staining patterns summarized by Table II. The frequencies of the immunofluorescent patterns for various antibodies are also given in Table II. Clearly, the interphase cells display the dominant staining patterns seen in Fig. 1.

Comparative phase contrast (not shown) and immunofluorescence micrographs (Fig. 1) indicate that the cognate antigens of these antibodies are arranged in distinctive patterns within the nucleus. Although some antibodies revealed staining juxtaposed to the nuclear membrane (e.g., AhNA 2, Fig. 1), as far as could be discerned, no cytoplasmic fluorescence was evident. Nor is the nuclear staining so diffuse as found for heterogeneous nuclear RNA-ribonucleoprotein antigens (26). The staining patterns are always highly reproducible within and between experiments.

The most readily interpretable staining is the specific decoration of metaphase chromosomes by AhNA-1 (Fig. 1). This antigen is present in two cell types and species (HeLa cells and rat skeletal myoblasts) since both cell types show identical patterns of staining of metaphase chromosomes (rat not shown). No other monoclonal antibodies tested stain mitotic chromosomes in this pattern (see Fig. 3).

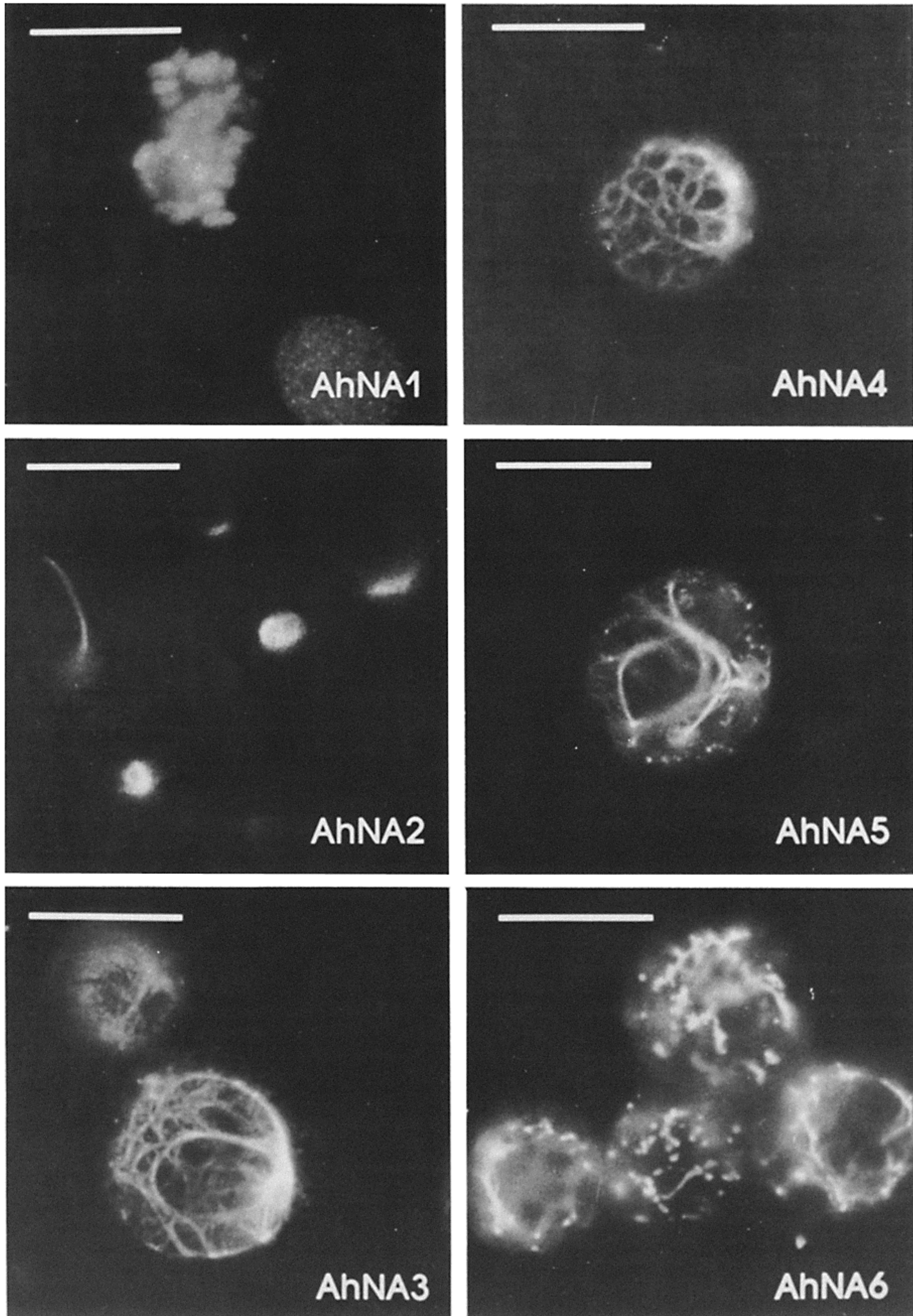

FIGURE 1 Indirect immunofluorescence photomicrographs of unsynchronized HeLa cells treated with mouse monoclonal antibodies to the HeLa TBPs. Bars, 10 μm.

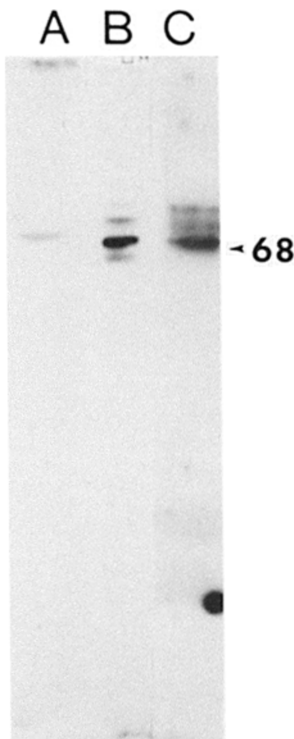

FIGURE 2 Autoradiogram of the electro-blot analysis of the binding of the monoclonal antibody AhNA-4 to HeLa cytoplasmic (lane *A*), TBP (lane *B*), and nuclear matrix (lane *C*) proteins. For each sample, 150 μg total protein was separated in 11% polyacrylamide-SDS gel slab, transferred electrophoretically to nitrocellulose, incubated with the antibodies, and processed for autoradiography as described under Methods. Exposure was for 18 h in the presence of an intensifying screen. Arrowhead indicates molecular weight × 10^{-3}.

A second staining pattern is shown by AhNA-2, which binds to a single spot within the nucleus or appears in a crescent shape beneath the nuclear envelope (Fig. 1). It is reminiscent of the chromocenter of *Drosophila* chromosomes (27). Whether this antibody detects a chromocenter or heterochromatin-associated protein(s) near the inner nuclear membrane in HeLa cells remains to be established. Note that this spot is not coincident with nucleoli, which appear as dark bodies in phase contrast micrographs (not shown).

Monoclonal antibodies AhNA-3, 4, and 5 reveal somewhat similar matrixlike structures within the nucleus (Fig. 1). AhNA-3 and 4 give positive ELISA reactions with preparations of HeLa nuclear matrix (Table I). They also detect the same small set of TBP and nuclear matrix proteins on "Western" gels (Fig. 2; gel for AhNA-3 gave a similar blot, pattern not shown). The same three polypeptides in TBP and nuclear matrix preparations are recognized by AhNA-4. The predominant polypeptide is ~70,000 daltons (Fig. 2). Three major polypeptides of the rat liver nuclear matrix are between 60,000 and 70,000 daltons (16). These antibodies may detect an antigen that is shared by a few polypeptides. Alternatively, they may detect different antigens on the same set of polypeptides. The fact that AhNA 3 and 4 react with nuclear matrix antigens in ELISA (Table I) and "Western" blots (Fig. 2) raises the possibility that the observed staining in Fig. 1 corresponds to the nuclear matrix. Monoclonal antibody AhNA-5 is clearly different form AhNA-3 and 4. It does not form an immune complex with either TBP or nuclear matrix proteins on "Western" blots or TBP solubilized by any of our current methods. Thus, we have not determined the molecular weight of its antigen. The matrixlike pattern that AhNA-5 shows is always more delicate than that made apparent by AhNA-3 and 4 (Fig. 1). Thus, two different matrixlike patterns are made visible by these antibodies.

The unusual immunofluorescent configurations are not artifacts of the formaldehyde cell fixation procedure, as shown by the following. First, an alternate fixation procedure using acetone gives identical immunofluorescent patterns. Second, the AhNA-1 specific staining of metaphase chromosomes is undisturbed by fixation, thereby constituting a strong control for chromatin staining. Furthermore, the absence of staining by secondary antibody alone also indicates that these monoclonal antibodies are specific for HeLa chromatin nonhistone proteins.

Finally, AhNA-6 reveals a "patchy" nuclear distribution of its antigen. This pattern may be that of a heterochromatin-specific polypeptide. Its immunofluorescence pattern is definitely not like the matrixlike pattern given by AhNA-3, 4, or 5. Although many cells have been examined by indirect staining with AhNA-6, none have threadlike, criss-crossing structures shown by AhNA-3, 4, or 5. This antibody has not been extensively studied because the hybridoma line has not yet been stabilized.

Cell Cycle and Nuclear Immunofluorescence

Physical and chemical alterations in chromatin structure are known to occur at different stages of the cell cycle in HeLa (20, 28, 29). Cytological evidence, using the fluorochrome quinacrine, suggests a continuous chromosome cycle throughout interphase in human (30) and mouse (31) cells. In order to explore the relationship between chromatin organization and the cell cycle, we have used three of the monoclonal antibodies to determine whether the structural organization of their antigens within the nucleus changes as HeLa cells progress through the cell cycle.

Fig. 3 illustrates that the organization of the antigens of AhNA-1, 2, and 3 changes dramatically as cells progress through the cell cycle. Although these antibodies reveal distinctly different patterns, one common feature observed is the fluorescent patterns that cover the entire cell during mitosis, for example, panels for AhNA-2 and 3 in Fig. 3. Because the nuclear envelope breaks down at metaphase, one should not be surprised to find that nucleoplasmic proteins are released into the cytoplasm at this stage. The diffuse staining is not an artifact because AhNA-1 shows localized fluorescence at mitosis. We interpret the staining pattern of AhNA-2 and 3 at mitosis in the Discussion. In the following, we note particular features revealed by AhNA-1, 2, and 3.

Fig. 3 (top panel) shows the immunofluorescent patterns obtained with AhNA-1. Cells in G1 have a distinctly granular pattern throughout the nucleus. At mitosis, fluorescence is highly localized in patterns that suggest condensed chromosomes which reverses to a granular pattern again in G2 (see also Fig. 1). We interpret these results to mean that the antigen of AhNA-1 is bound to both interphase chromatin and condensed chromosomes.

Monoclonal antibody AhNA-2 recognizes a tight-binding protein which is regionalized in the nucleus. G1 stage cells show a single, circular structure which often has the appearance of a stack of carefully coiled threads (Fig. 3, middle panel). In some instances, these threads radiate outwardly to lie beneath the nuclear envelope. At mitosis, the pattern is granular, covering the entire cell, and in G2 cells, one sees the return of the G1-like bright fluorescent center within the nucleus. This staining pattern is highly reproducible. Between 60 and 80% of cells in the synchronized population show the characteristic bright spotlike fluorescence of G1 and G2 cells.

Although we cannot yet confirm this inference, the immunofluorescence pattern of AhNA-2 suggests that its antigen is a part of heterochromatin and that the dense bright, coiled

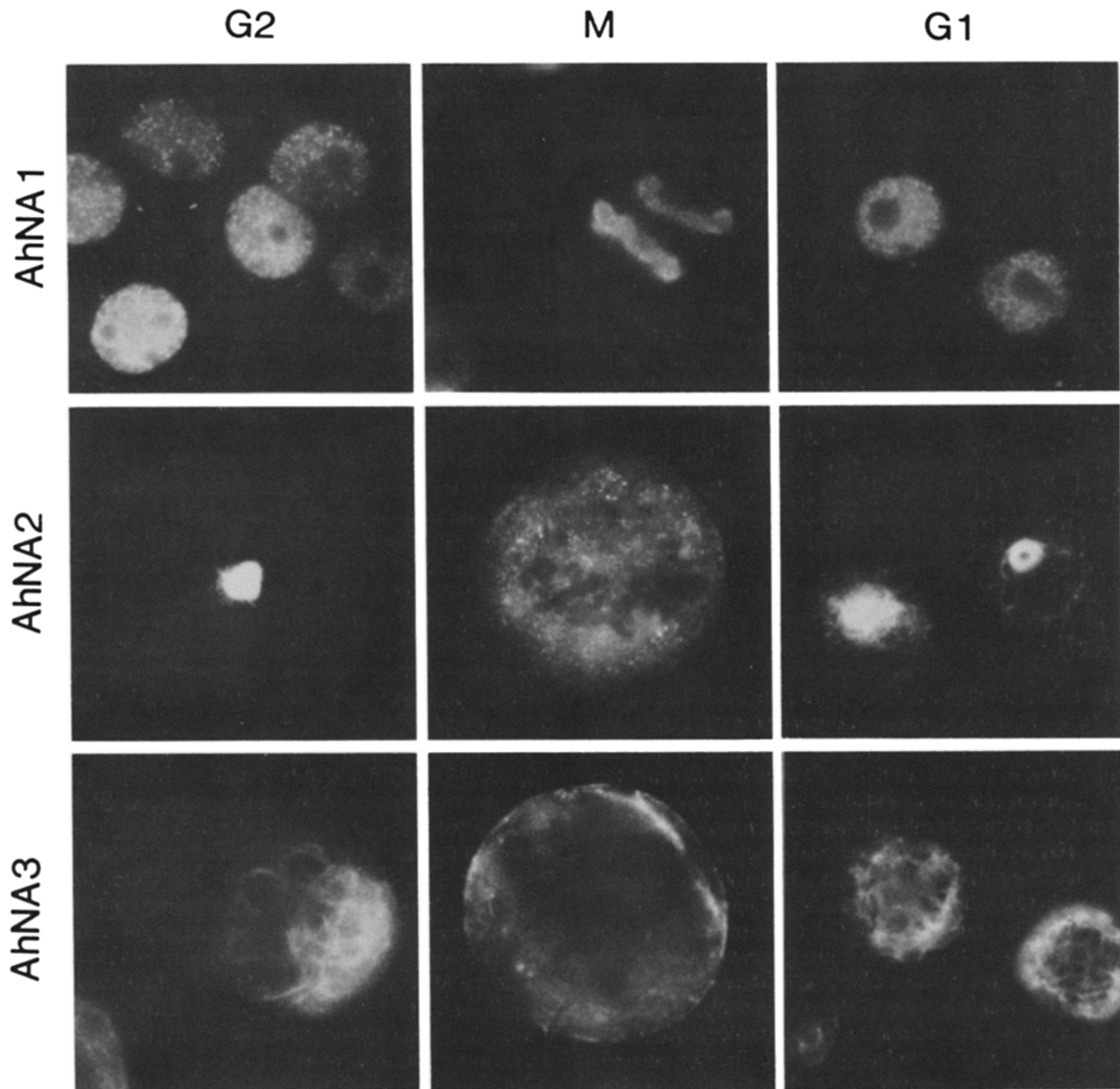

FIGURE 3 Changing patterns of nuclear distribution of the cognate antigens as revealed by indirect immunofluorescence of synchronized HeLa cells treated with monoclonal antibodies: AhNA-1 (*top* panel), AhNA-2 (*middle* panel), AhNA-3 (*bottom* panel). Synchronized cells were harvested at 5 h (*S*), 8 h (*G2*), 10 h (M), and 13 h (early *G1*) after release from the thymidine block (20). Note: the micrograph for AhNA2-G1 has been spliced to juxtapose two cells in the same view.

structure is a "chromocenter," as defined by Natarajan and Gropp (32). We are unaware of any immunofluorescence studies that have directly visualized a chromocenter of human cells. However, earlier cytological studies using Feulgen staining and fluorochrome dyes suggested that chromocenters are formed by the fusion of constitutive heterochromatin in the mouse (32, 33). A disperse, faintly quinacrine fluorescent pattern in late G1 to bright fluorescent granular pattern in late G2 and prophase of constitutive heterochromatin (=chromocenters) in HeLa and mouse fibroblast nuclei has been shown to occur as cells progress through the cell cycle (30, 31). Furthermore, the number and size of chromocenters seem to vary with the cell cycle stage, being an average of 13 chromocenters per nucleus in late G1 to 35 chromocenters per nucleus in late G2 to prophase in the mouse (31). The sequence of the bright spotlike immunofluorescence in *G2* to granular pattern in *M* and return to dense bright structure in *G1* for the AhNA-2 antigen (Fig. 3, *middle* panel) suggests possible alterations in the functional states of constitutive heterochromatin, i.e., the chromocenter, if, indeed, this chromatin arrangement can be equated with the quinacrine staining appearance of chromocenters (30–32).

Fig. 3 (*bottom* panel) illustrates the staining pattern of AhNA-3. In both G1 and G2 cells a filamentous, matrixlike pattern is seen in the nucleus (see also Fig. 1). This pattern is reproducible and is displayed by 70% or more cells within an unsynchronized population (Table II). Staining of the cytoplasm does not occur in G1 or G2 cells. In contrast, cells in mitosis show a diffuse, but slightly granular, pattern of fluorescence that includes the entire cell. As noted above and in

the Discussion, the antigen of AhNA-3 may be released within the cytoplasm when the nuclear envelope breaks down.

We note here as a word of caution that S-phase cells obtained by the double-thymidine block synchronization procedure displayed an abnormal diffuse fluorescence over the total cell, regardless of the antibody used. This may be due to the known cytotoxic effects of the high thymidine concentrations used for induction of cell synchrony, which have been shown to cause reversible unbalanced growth, abnormal cell metabolism, and nuclear damage (34–37).

DISCUSSION

Only a few studies have used monoclonal antibodies as reagents to identify and survey the spatial distribution of chromosomal proteins (38, 39, 41, 42, 44). Our study differs from the previous ones in two ways. First, we have focussed specifically on chromatin-associated proteins that remain bound in a DNA-protein complex in the presence of 2.5 M NaCl-5 M urea. The significance of these proteins was discussed in the Introduction. Second, we have examined the subcellular localization of these antigens throughout the cell cycle.

The antigen identified by AhNA-1 is clearly associated with chromatin. The entire metaphase chromosome is visualized by immunofluorescent staining with AhNA-1. This is in contrast to regional staining, i.e. staining of only the termini, as observed by Will and Bautz (27) who used a polyclonal antiserum specific for heterochromatin. The cognate antigen of AhNA-1 is organized in the chromosome in a way that makes it readily accessible to the antibody. This antigen is unlike the one identified by Turner (38), which becomes accessible to the antibody only after salt washing of nuclear preparations. AhNA-1 does not cross-react with HeLa core histones. Therefore, the even distribution of fluorescence along the metaphase chromosome does not result from reaction of the antibody with core histones. The granular nuclear staining in other cell cycle stages suggests that this antigen is present in chromatin throughout the cell cycle. We have shown that the antigen is not DNA, core histones, or HMG proteins (Table I). Thus, it appears that a new component of chromatin has been identified. This antigen is also present in rat chromatin.

Other workers have shown that antigens present in total nonhistone protein preparations are widely distributed along the entire length of individual polytene chromosomes (40). In one case, a cognate antigen of a monoclonal antibody that is specific for a nonhistone protein is known to be localized in regions of active or potentially active genes (41). The antigen (AhNA-1) we identify may similarly be localized in active (or inactive) gene regions, but we cannot test this directly by immunofluorescence methods because of cytological limitations inherent to nonpolytene chromosomes. Another alternative that we hope to test is that this antigen is part of the chromosome scaffold (17).

The pattern of immunofluorescence given by AhNA-2 and AhNA-3 is apparently random in mitotic cells. This may mean either that the antigen is released from tight complexes with DNA during mitosis, or that it remains associated at only a few regions of each chromosome and these regions are distributed randomly in chromosomes at mitosis. We believe the first possibility is more likely because the area of diffuse staining is quite large, covering the entire cell.

The antigen identified by AhNA-3 appears to be organized in the nucleus in a way different from the two discussed above. Its organization during G1 and G2 resembles that of a nuclear matrix. In addition, preparations of nuclear matrix contain the cognate antigens of AhNA 3 and AhNA 4. It is of interest to note that this structure disappears during mitosis, a period during which the breakdown of the nuclear envelope and presumably the nuclear matrix occurs. By early G1 the structure reappears. Apparently, therefore, the antigen is not bound to chromosomes at mitosis even though it clearly is a component of interphase chromatin. These results strongly suggest that the matrixlike structures visualized by antibodies AhNA-3 and AhNA-4 (Figs. 1 and 3) may constitute the nuclear matrix.

The contemporary view of chromosome structure and function is dominated by our considerable knowledge of the biochemical properties of histones and the high-mobility group of nonhistone proteins. This is largely because these proteins are abundant and can be isolated in native form. Furthermore, well defined functional assays are available for analysis of histones and HMG proteins. Bulk nonhistone chromosomal proteins are also thought to have important regulatory and structural roles. However, the regulatory proteins among these are probably present in much smaller amounts, and an almost total absence of suitable functional assays makes them quite refractory to conventional biochemical investigation. Thus, the immunological approach using monoclonal antibodies that we and others (38, 39, 41, 42) have begun will provide an effective method for accessing the functions of this important class of chromosomal proteins. Indeed, our use of such antibodies to study changes in the distribution of specific nuclear antigens as HeLa cells traverse the cell cycle clearly indicates the advantage of the immunological approach.

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