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Monoclonal Antibody with Specificity to a Conserved Epitope in the C-Terminal Domain of Histone H1 Variants

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ABSTRACT. **A monoclonal type M-immunoglobulin (IgM) was generated in mice against a nuclease-urea extract of HeLa metaphase chromosomes. This antibody stains metaphase chromosomes from a variety of mammalian cultured cell types by indirect immunofluorescence. Antibody 12C7 reacts by western transfer technique with histone H1 in all the cell lines tested. The antibody cross-reacts with H1, and $H1^0$ in human cells. Proteolytic digestions of H1 suggest that the epitope is localized in the carboxy-terminal domain of the histone H1 molecule. Digestion with trypsin demonstrates that the antibody 12C7 does not react with the globular domain of histone H1. The C-terminal domain of H1 subtypes therefore seems to have a conserved determinant which does exist in H1, $H1^0$, and probably in H5. This antibody has applications in studying the role of that domain of H1 in processes like chromosome condensation and variations in chromatin structure which influence gene expression.**

The lysine-rich class of histones are a group of chromosomal proteins that are involved in the establishment and maintenance of higher-order structures in chromatin (1, 2, 10). On the basis of primary structure, it is clear that H1 are a unified family of proteins. The overall composition of the H1 complement varies considerably among species and even among tissues within a given species. Thus, $H1^0$ and H5 occur predominantly in cells that are mitotically inactive and may have similar functions (2). By proteases digestion studies there are three structural domains of histone H1 (3). For calf thymus, the defined domains are: 1) a basic residue at the N-terminus; 2) a central globular domain; and 3) a basic charged residue at the C-terminus. The globular domain is conserved between species and H1 subtypes, but cannot condense chromatin on its own. Therefore the N-terminal and C-terminal tail responsible for the variant of the molecule must also be involved in the process of chromatin condensation. In particular, that role fits well with the long basic C-domain of H1 but less with the shorter N-tail (10).

Antisera to histones and to a variety of non-histone chromatin proteins have been used for the study of eukaryotic chromosomes by immunofluorescence and electron microscopy (5, 9, 15, 16). Immunomicroscopy techniques can provide detailed information on protein distribution which can be used to deduce the probable roles of individual proteins in the maintenance of chromosome structure and function. For immunomicroscopic and biochemical analysis, monoclonal antibodies have several advantages over conventional antisera. Among others, those include improvements in the specificity of antibody, greater reproducibility, and localization of the protein sequence (epitope) responsible for a determined function.

We have used an immunological approach to develop probes to study the role of nuclear proteins in some aspects of mitosis, including chromosome condensation. This report describes a monoclonal antibody, which reacts strongly with mitotic chromosomes and interphase nuclei in a variety of mammalian cells. The antigen is identified as histone H1, and data is presented on the localization and identification of the epitope.

MATERIALS AND METHODS

Preparation of antigen and antibody production. HeLa cells were grown in suspension culture in McCoy's 5a medium supplemented with 7% fetal calf serum. Cells in exponential growth were synchronized by adding 2.5 mM thymidine to the medium for 18 hours, and then treated with colcemid at 0.06 μg/ml in fresh medium for 16 hours. Mitotic index of 98% was commonly obtained as determined by phase-contrast microscopy. The hexylene glycol method for metaphase chromosomes preparation was used, starting with 10^8 mitotic cells (19). Chromosomes were further purified by a glycerol

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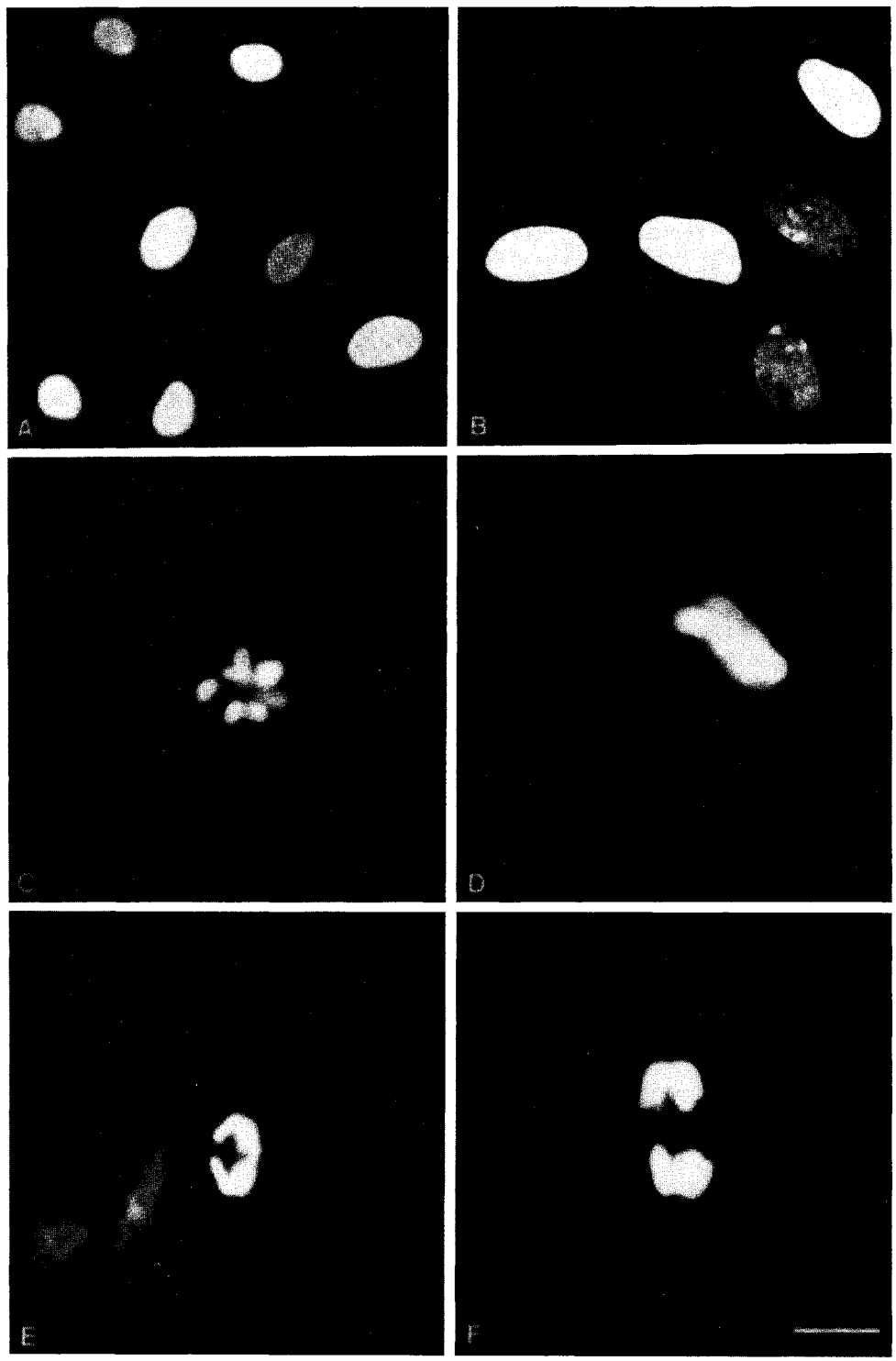

Fig. 1. Profile of 12C7 antigen localization on various preparations of Indian muntjac cultured cells. Panels A–B show immunofluorescence in interphase cells. Some nuclei show very bright staining, while others are slightly detectable. Following its nuclear deposition 12C7 antigen associates with mitotic chromosomes during prometaphase (C), metaphase (D), anaphase (E), and telophase (F). Bar equals 20 μm.

gradient (8). Purified chromosomes were incubated in 10 mM Tris-HCl pH 7.1, 2 mM $CaCl_2$, 1 mM PMSF (buffer A) containing 200 U/ml of micrococcal nuclease (MNAse) for 1 hour at 37°C. Later, the suspension was centrifuged in a Beckman JA-20 rotor at 5,000 rpm for 10 mim at 4°C. The pellet was resuspended in 10 mM Tris-HCl pH 7.1, 1 mM EDTA, 1 mM PMSF (buffer B) containing heparin at 2 mg/ml and incubated at 4°C for 1 hour. The suspension was centrifuged as above and the pellet was resuspended in buffer B containing 3 M urea and incubated for 1 hour at 4°C. The preparation was finally centrifuged at 10,000 rpm for 15 min at 4°C. The final supernatant was used as the source for immunizations.

BALB/c mice were immunized intraperitoneally with crude urea extracts prepared from HeLa metaphase chromosomes as described above. Approximately 400–500 μg of total protein was used per injection emulsified in Freund's adjuvant.

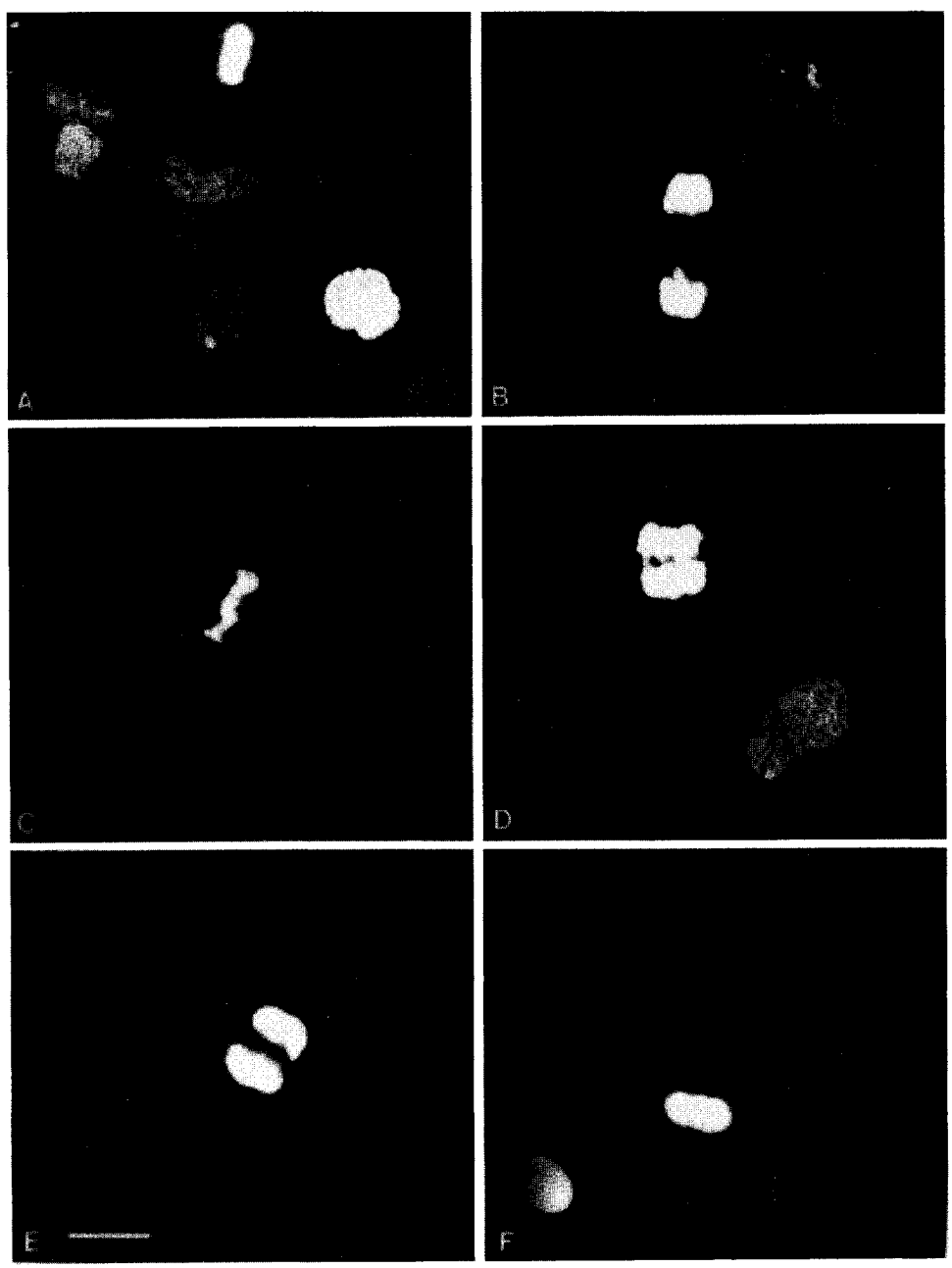

Fig. 2. Immunofluorescence microscopy of mitotic mammalian cells with anti-H1. Note the bright staining pattern associated with condensed chromosomes of HeLa (A), Indian muntjac (B), PtK (C), CHO (D), BHK (E) and 3T3 cells (F). Again like in Figure 1 staining in interphase nuclei is much lower than in mitosis. Bar equals 20 μm.

The mice were boosted every 4 weeks for three months. Blood samples obtained from the tail were used in a immunofluorescence screening assay for chromosomal antigens. Spleen cells from the selected mouse were removed, dissociated, and spleenocytes were fused with mouse myeloma SP2/0 cells using standard procedures as described (12). Hybridoma cultures were assayed by immunofluorescence on HeLa cells cultured on sterile coverslips. Cultures were fixed with cold methanol for 10 min at −20°C. The immunofluorescence procedure was similar to that described in a previous paper (20). Hybridomas found by the assay to secrete the desired antibody were harvested and cloned three times by limiting dilution. The hybridoma clones were then grown up in flasks and used as the source of monoclonal antibodies for subsequent experiments. They were also amplified as ascitic tumors in BALB/c mice primed with 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane). After 7–14 days, the ascitic fluid was collected, cleared by centrifugation and stored at −80°C.

Electrophoresis and Western Blots Analysis. Polyacrylamide gel electrophoresis (PAGE) in the presence of SDS was performed using the procedure of Laemmli (13). Proteins were electrophoretically transferred onto nitrocellulose filters (18) using a Bio-Rad trans-blot apparatus. Filter strips were incubated with 3% BSA dissolved in 10 mM Tris—150 mM NaCl saline buffer (TBS) pH 7.5 for 4 hours at room temperature to block non-specific binding to the paper. Then, filter paper was incubated for 4 hours to overnight at room temperature with antibody 12C7 diluted 1 : 400 in TBS and washed with TBS containing 0.05% Tween, three times for 15 min each. Finally filter was incubated for 1–2 hours with affinity purified peroxidase conjugated goat antimouse IgM, followed by several washes in TBS—Tween and developed with chloronaphthol (20).

Histone H1 extraction and cleavage. Either tissue cultured mammalian cells or human placenta nuclei were extracted with 5% perchloric acid (PCA). The extract was precipitated with trichloroacetic acid (TCA) at 20%. The precipitate was washed with acetone-HCl and acetone and resuspended in appropiate buffer and stored at −70°C until further used.

Purified human H1 was cleaved with chymotrypsin at 1 mU/μg H1 at room temperature for 20 min in 5 mM Tris-HCl pH 7.5, to yield peptides corresponding to N-terminal and C-terminal domains (7, 21). To remove a short N-terminal residue of H1, human placenta H1 was incubated with submaxillary protease (7, 21) at 2 mU/μg H1 at r.t. for 20 min in 50 mM Tris-HCl at pH 7.5. Also, H1 was digested with N-bromosuccinimide (NBS) at 250 : 1 molar ratio in 50% acetic acid (7, 16). After 4 hours, fresh NBS was added and the reaction was terminated by precipitation with acetone. H1 was also digested with trypsin (1, 7) for 120 min at r.t. at 1.5 mU/μg H1 in 50 mM Tris, 200 mM NaCl pH 7.5, and the reaction was terminated by adding 5 mM phenylmethylsulfonyl fluoride (PMSF). This last incubation resulted in the production of the globular fragment of the molecule H1 (1, 7). Digestion with thrombin was achieved at 15 miliunits/μg for 2 hours to overnight at 37°C to produce N-terminal and C-terminal peptides (3, 7). Digestion with carboxypeptidase A was done initially at 0.25 U/ml in 10 mM Tris, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT pH 7.5 for 90 min. The amount of enzyme was increased with the digestion time to generate shorter H1 peptides.

RESULTS

Immunofluorescence staining of mammalian cell lines with monoclonal antibody 12C7. By indirect immunofluorescence the antibody 12C7 gave a bright staining of methanol-fixed cultured cells (Fig. 1). Staining is restricted to nuclei in interphase cells and to chromosomes in mitotic cells. These localizations suggest a chromatin —bound antigen throughout the cell cycle. The staining is slightly decreased when cells were fixed

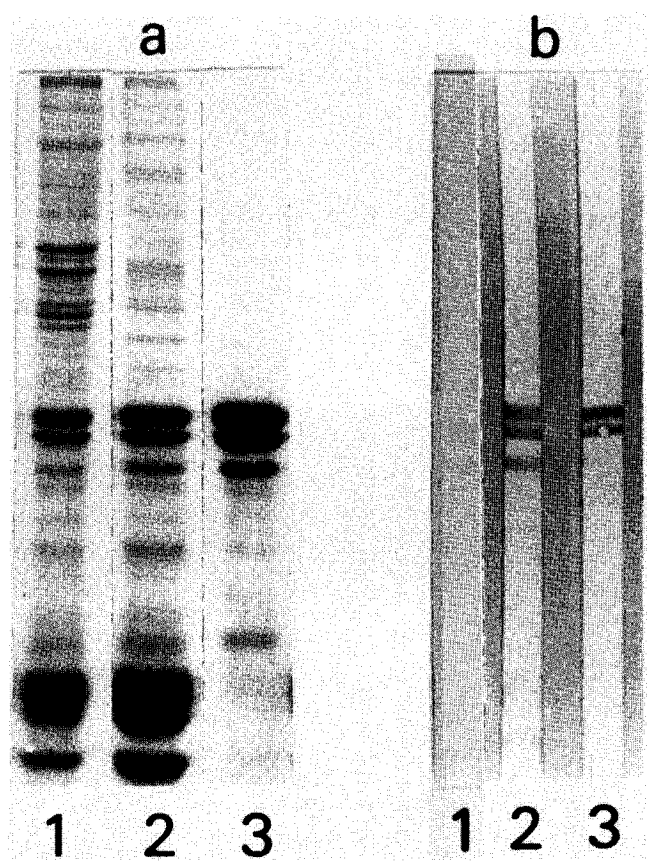

Fig. 3. Immunoblot of human cells extracts with anti H1. a) Coomassie blue staining of whole human placenta nuclei proteins extracted in SDS-Laemmli buffer (1), HCl extracted proteins from placenta nuclei (2) and perchloric acid isolated histone H1 from placenta nuclei (3). b) Two major bands, 32 kD–30 kD, are revealed upon probing with antibody 12C7. Those polypeptides correspond to histone H1. A minor one also reactives with the antibody corresponds to $H1^0$ on lanes 2 and 3.

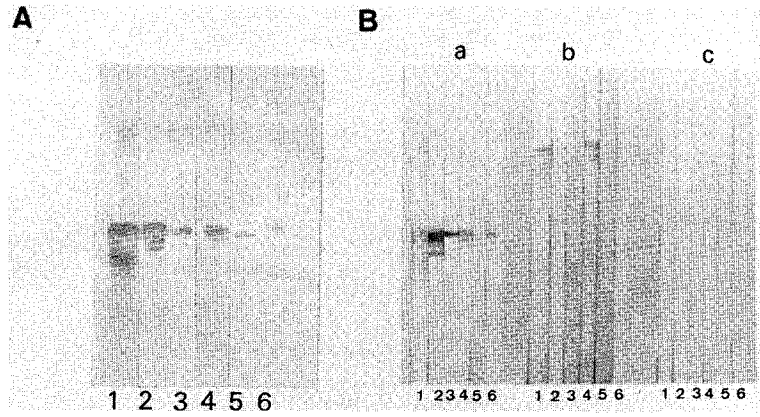

Fig. 4. Immunoblot of isolated histone H1 from mammalian sources with monoclonal 12C7. Proteins were separated on a 12.5% polyacrylamide-SDS gel transferred to nitrocellulose paper, and reacted with monoclonal 12C7 as described under "Materials and Methods". Panel A): Coomassie blue staining of proteins isolated by perchloric acid extraction and TCA precipitation from different mammalian cell lines. 3T3 (lane 1), HeLa (lane 2), PtK (lane 3), CHO (lane 4) Indian muntjac (lane 5) and BHK (lane 6). Panel B): Immunoblots with monoclonal 12C7 and controls. Lanes 1–6 in a) incubated with anti 12C7. Lanes 1–6 in b) incubated with another monoclonal as a negative control. Lanes 1–6 in c) incubated with preimmune serum.

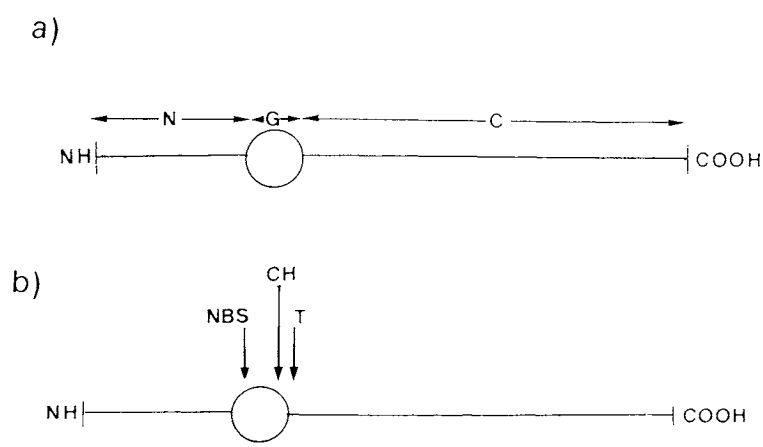

Fig. 5. Representation of H1 molecule. (a) Includes amino-terminal (N), globular region (G) and carboxyl-terminal (C). In (b) is shown cleavage sites by N-bromo-succinimide (NBS), chymotrypsin (CH) and thrombin (T) as described in reference 7.

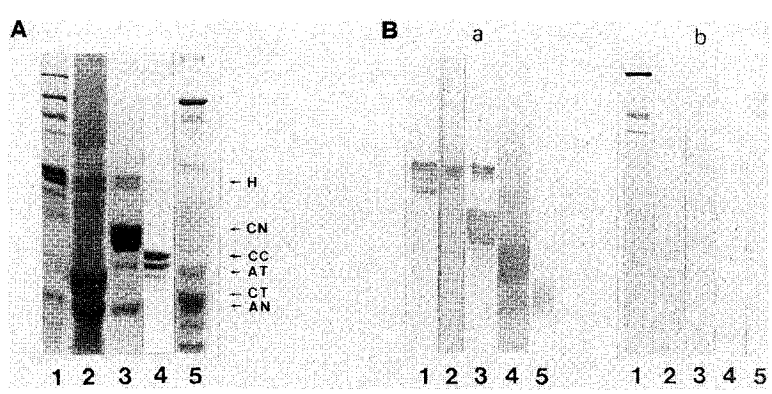

Fig. 6. Immunoreactivity of histone H1 peptides with antibody 12C7. Peptides of purified H1 were prepared as described under "Materials and Methods", separated on 15% SDS-PAGE and transferred to nitrocellulose. One set was stained with Coomassie blue (panel A) and two identical sets were incubated with 12C7 antiserum (panel B, a) or antiserum control (panel B, b). Lane 1, molecular weight standard including histone H1 (arrow H); lane 2, HCl extracted proteins from human placenta nuclei; lane 3, N-bromosuccinimide peptides (CN, carboxyl-terminal; AN, amino-terminal); lane 4, chymotrypsin peptides (CC, carboxyl-terminal); lane 5, thrombin peptides (AT, amino-terminal; CT carboxyl-terminal).

with formaldehyde, possibly due to masking the antigen caused by the cross linking of DNA and histones produced by this fixative. The variability of staining observed in interphase nuclei (Fig. 1, 2) was presumably due to differences in antigen accesibility rather than differential distirubution of antigen within the chromatin. However, the possibility can not be ruled out that the epitope in H1, recognized by monoclonal 12C7 is a posttranslational modification cell-cycle regulated (see discussion). Cell-cycle regulated genes and posttranslational phosphorylation and acetylation are common events for the histone family (2, 4, 17). Further experiments are needed to resolve this possibility. Figure 2 shows mitotic cells from human (A), deer (B), kangaroo rat (C), hamster (D), bovine (E), and mouse (F) origin, stained with monoclonal 12C7 by indirect immunofluorescence. In all cases metaphase chromosomes in mitotic cells were stained very brightly. Some pictures in Figure 2 also showed staining of interphase nuclei as is the case for HeLa (A), Indian muntjac (B), CHO (D), and 3T3 (F).

Identification of 12C7 antigen by immunoblotting. Because of the immunofluorescence behaviour of the 12C7 antigen, a main chromosomal associated polypeptide seems to be the obvious candidate for the monoclonal reactivity in mammalian cells. This was confirmed by the reactivity observed by western blots. Major bands in the 31 kD region were identified in whole HeLa cells extracts (Fig. 3). These bands presented a similar electrophoretic mobility and pattern to histone H1. Based in these preliminar identification of the 12C7 antigen, we decided to demonstrate its similar-

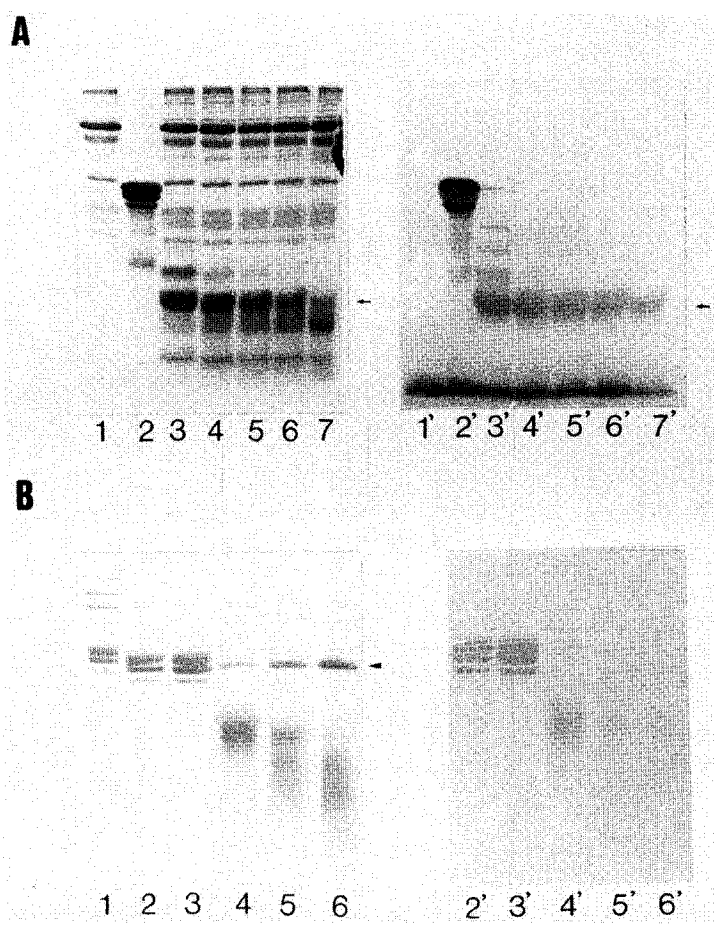

Fig. 7. Immunoblotting of time cleavage of histone H1 with thrombin and carboxypeptidase. Peptides of purified H1 were prepared as described under "Materials and Methods", separated on 15% SDS-PAGE, and transferred to nitrocellulose. One set was stained with Coomassie blue (lanes 1–7 in panel A for thrombin, and lanes 1–6 in panel B for carboxypeptidase), and identical sets were incubated with 12C7 antibody. In A: lanes 1 and 1′ thrombin pattern; lanes 2 and 2′ isolated human histone H1; lanes 3 and 3′ H1 digested for two hours; lanes 4 and 4′ for 4 hours; lanes 5 and 5′ for 6 hours; lanes 6 and 6′ for 8 hours; lanes 7 and 7′ overnight digestion. In B: lane 1 molecular weight markers; lanes 2 and 2′ isolated human histone H1; lanes 3 and 3′ H1 digested for 90 minutes; lanes 4 and 4′ for 120 min.; lanes 5 and 5′ 180 min.; lanes 6 and 6′ 240 min.. Arrow in A indicates the carboxyl-terminal region of H1 molecule. Arrow in B indicates the carboxypeptidase enzyme pattern.

ity to the basic histone H1. An important criterion for identification of an H1 protein is its ease of extraction from nuclei with 5% PCA (11). This procedure is known to remove H1 and a small amount of non histone proteins while the inner histones and bulk of non-histones remain associated with the DNA. Figure 4A illustrates the separation of a total PCA extract from different mammalian nuclei examined by gel electrophoresis. It is evident that the majority of PCA-extractable proteins migrates in a few closed stained bands, although differences are appreciable between species. The major protein band(s) at the 31 kD molecular weight range constitutes 90% of the total stained materials. An immunoblot of the same SDS-PAGE H1 isolation, developed with monoclonal 12C7 and peroxidase-goat antimouse immunoglobulin, is presented in Figure 4B. The monoclonal reacts with all histones H1 isolated from different mammalian cell lines, indicating the presence of a common epitope in those molecules, as shown before by others by sequencing experiments (6). Moreover, this Figure shows that the antigen was soluble in 5% PCA and its migration coincided with the migration observed for H1 (H1 standard from Sigma Chem. Co. was used as a control, data not shown). The antigen appeared as a doublet or triple bands, the mobility of which coincided with the mobility of H1 subtypes described previously (2, 3). Also, the epitope recognized by the monoclonal antibody on the H1 molecule is conserved among mammals. This result confirms the immunofluorescence results of tissue culture cells.

In order to identify the domain in the H1 molecule that was recognized by the monoclonal 12C7, we carried out immunoblotting experiments using peptides of human histone H1 that were prepared by digestion with a variety of proteolytic enzymes essentially as described (7). A description of expected H1 peptides generated with the reagents used in this paper, have been reported previously be several authors (3, 5, 7, 9, 16, 21). Figure 5b shows a scheme of several proteases cleavage sites on the H1 molecule. Our results on H1 protease digestions originates peptide patterns that were coincident with those described by others, when examined by SDS-PAGE (Fig. 6, 7, 8). The results indicate that the H1 antigenic domain lies in the C-terminal half of the molecule (Fig. 6B, 7A, B, and 8). Reactivity was restricted to peptide 107–193 (C-terminal region) as shown by immunoblots after proteases digestions (see Fig. 6B). No reaction was detected on the N-terminal region of H1 with monoclonal 12C7 as indicated after treatment with NBS (peptide 1–73) and chymotrypsin (peptide 1–107) on Figure 6B, a (lanes 3 and 4). Cleavage of the H1 molecule with carboxypeptidase A at the C-terminal region confirms those results as shown on Figure 7B (lanes 5 and 6).

Thus we concluded that this antibody is specific for the carboxyl-terminal domain of H1, and that the epitope is conserved in several histone H1 subtypes, and that it is expressed in different mamalian cell lines.

DISCUSSION

By use of immunological staining in fixed cells and electrophoretic transfer onto nitrocellulose filters we have shown that monoclonal antibody 12C7 recognizes

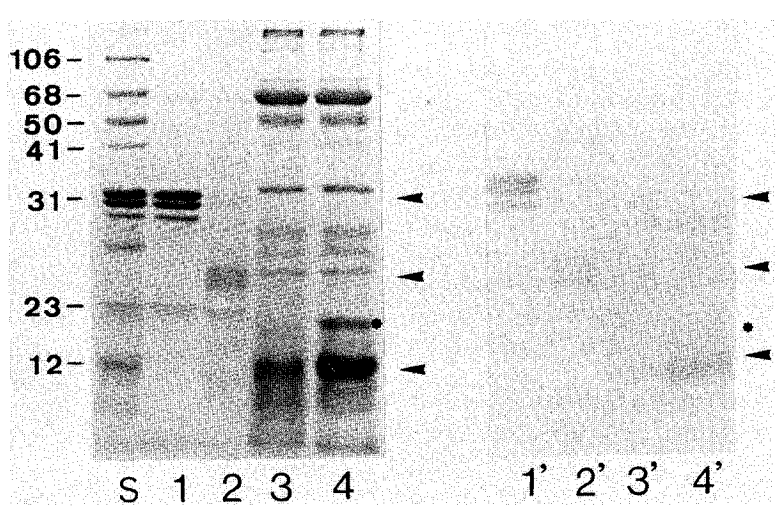

Fig. 8. Immunoblot of H1 peptides generated by double enzymatic cleavage. Peptides of purified H1 were prepared as described under "Materials and Methods" separated on 15% SDS-PAGE, and transferred to nitrocellulose. One set was incubated with antibody 12C7 and an identical set was stained with Coomassie blue. Lane 1 isolated human histone H1; lane 2 NBS peptides; lane 3 thrombin cleavage of NBS peptides; lane 4 thrombin cleavage of H1. Note the absence of N-terminal of H1 in lane 3 and its presence in lane 4 (*). Antibody 12C7 reacts with whole H1 molecule (lane 1'), NBS peptides (lane 2'), C-terminal (lane 3') but not with N-terminal (lane 4').

an antigen with the same mobility in SDS-PAGE as the histone H1. The conclusion that the antigen is H1 is consistent with several of its properties revealed by indirect IF microscopy and biochemical approaches, namely 1) the antigen is exclusively nuclear in interphase cells; 2) it is present on mitotic chromosomes; 3) the antigen is conserved, being expressed in all the mammalian cell types so far tested; 4) the antigen is extracted from nuclei by 5% perchloric acid and precipitated by 20% TCA; and 5) its molecular weight is in the range of 32–30 kD in SDS-PAGE.

Multiple reports have shown that the globular domain of histone H1 is highly conserved, whereas the flanking regions have accumulated more changes. However, short regions within the molecule do show strong homology between H1 subtypes (16). So, all histones H1 variants contain a basic hydrophobic carboxy-terminal tail of 110 amino acids which probably is capable of binding to the phosphate backbone of DNA. In addition to a well defined adenine binding site on the H1 molecule (2), several nucleotide binding sites have a lysine residue towards the carboxy terminus (2, 10), and several other putative phosphate binding sites in H1 have been described towards the carboxy terminus (1, 2, 3, 10). These consensus sequences have been described in H1 molecules from human, chicken, rabbit, nematodes, sea urchins and murine (2, 10, 14). The C-terminal region has been shown to be specially effective in condensation. According to Allan *et al.* (1) and many others, the C-terminal domain remains the most likely region for the condensation of the chromatin and fits it well for the ionic interactions with the DNA. Overall, histone H1 is a ubiquitous constituent of both interphase and mitotic chromatin in somatic cells. In view of this, the non uniform IF staining observed in some interphase cells with monoclonal 12C7 is of some interest. Several explanations for these observations can be considered, namely, that the antibody is directed against a posttranslational modified form of H1 or that the accessibility of the antigen varies within different stages of interphase cells. A definitive answer to the first possibility will require detailed biochemical characterization of the epitope on the H1 molecule and analysis of the influence of acetylation, phosphorylation and other modification on the binding to monoclonal 12C7. Such work is in progress.

The present study clearly demonstrates that various histone H1 variants possess a common domain in the C-terminal region of the molecule. It is not possible, however, to determine on the basis of the present results where the epitope is localized in the C-tail and if it corresponds to one of the well characterized phosphate binding domains. The monoclonal 12C7 described in this paper will prove to be a useful probe for analyzing variations in chromatin structure (condensation) which influence gene expression.

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