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High Mobility Group Chromosomal Proteins Isolated from Nuclei and Cytosol of Cultured Hepatoma Cells Are Similar†

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ABSTRACT: Using sequential chromatography on columns containing immobilized double-stranded DNA and single-stranded DNA, we have purified a protein from the cytosol of an established line of cultured rat hepatoma cells that, by several criteria, is a high mobility group (HMG) protein. Analyses of DNA binding properties, electrophoretic mobilities, amino acid compositions, and immunochemical reactivities reveal that the cytosolic protein is the same protein as HMG-1 isolated from the purified chromatin of the same cell line. Thus, authentic HMG-1 appears to be at least partially responsible for the cytoplasmic fluorescence observed when mammalian cells are stained with fluorescent-labeled, affinity-purified antibodies against HMG-1 [Bustin, M., & Neihart, N. K. (1979) *Cell 16*, 181-189]. We suggest that HMG-1 can shuttle between nucleus and cytoplasm, perhaps in response to the nucleus' need for helix destabilizing proteins.

The high mobility group (HMG)[1] proteins are a class of nonhistone chromatin proteins that can be released from chromatin with 0.35 M NaCl and that are soluble in 2% trichloroacetic acid (Goodwin et al., 1973). The term HMG, which was first applied to proteins from calf thymus, refers to the high mobility that the proteins exhibit in an acid-urea gel electrophoresis system (Goodwin et al., 1973). The calf thymus HMG proteins have very distinctive amino acid compositions with high contents of both acidic and basic amino acid residues (Johns et al., 1975). Proteins that are similar in physical, chemical, or immunochemical properties to the calf thymus HMG proteins have been found in phylogenetically diverse organisms (Watson et al., 1977; Sterner et al., 1978; Spiker et al., 1978; Romani et al., 1979).

The HMG proteins were first isolated from chromatin, and they have therefore been thought of as nuclear components. Recently, however, Bustin & Neihart (1979) presented evidence that HMG-1 or proteins immunologically cross-reactive with HMG-1 occur in the cytoplasm of several types of cultured mammalian cells as well as in the nuclei of the same cells. That evidence was obtained by microscopic observations of

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[1] Abbreviations used: HMG, high-mobility group; HTC cells, hepatoma tissue culture cells.

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cells stained with fluorescently labeled, affinity-purified antibodies against HMG-1. Bustin & Neihart (1979) demonstrated that rat liver cytosol contains a protein of the same electrophoretic mobilities as HMG-1 in two gel systems but that cytosolic protein was not further characterized and was not completely purified.

In this paper we report the purification of HMG-1 from the cytosol of cultured hepatoma cells and compare the properties of that protein with the properties of nuclear HMG-1, whose isolation from the same line of cells was reported previously (Bidney & Reeck, 1978b).

Materials and Methods

Preparation of Immobilized DNAs. Salmon DNA (Sigma Chemical Co., Type III) was purified by several extractions with chloroform–isoamyl alcohol (24:1) and sheared in 1 mM EDTA (pH 7.0) for 1.5 min at full speed with a Sorvall Omnimixer. Double-stranded DNA was adsorbed to cellulose (Avicell microcrystalline) as described by Alberts & Herrick (1971). Double-stranded DNA–cellulose columns were treated with S_1 nuclease as previously described (Bidney & Reeck, 1978b). Single-stranded DNA, obtained by heat denaturation of purified and sheared salmon DNA, was attached covalently to agarose as described by Arndt-Jovin et al. (1975).

Isolation of HMG Proteins from Cultured Hepatoma Cells. Rat hepatoma tissue culture cells (HTC cells) were grown in suspension culture in Tricine-buffered Swim's 77 medium supplemented with 5% calf serum and 5% fetal calf serum (Thompson, 1979; Bidney & Reeck, 1978a). Cells were harvested by centrifugation for 10 min at 500*g*, washed with 50 mM Tris-HCl (pH 7.5)–0.25 M sucrose–3 mM $CaCl_2$, and collected by centrifugation. The cells were then homogenized (Potter-Elvehjem) in the same buffer supplemented with 1 mM phenylmethanesulfonyl fluoride and 1% Triton X-100. The nuclei, which remained intact during the homogenization, were collected by centrifugation for 10 min at 4000*g*. The nuclei were used as a source of HMG-1 with the following modifications of the procedure of Bidney & Reeck (1978b): chromatin was extracted with 0.35 M NaCl rather than with 0.75 M NaCl, and the double-stranded and single-stranded DNA columns were equilibrated to 0.2 M NaCl rather than to 0.05 M NaCl [see Isackson et al. (1979)].

As a source of cytosolic HMG proteins, we used the 4000*g* supernatant obtained, as described above, after homogenization of HTC cells. Typically $\sim 10^{10}$ cells were used in a preparation. The 4000*g* supernatant was poured off from the pellet of nuclei and centrifuged for 20 min at 20000*g*. The resulting supernatant was dialyzed for 20 h against a large excess of 0.2 M NaCl–1 mM Tris-HCl (pH 7.5), and the dialysate was clarified by centrifugation for 30 min at 20000*g*. The clarified sample was adjusted to 40% saturation by adding solid ammonium sulfate. The precipitate was collected by centrifugation for 10 min at 10000*g*. The supernatant was dialyzed against several changes of 0.2 M NaCl–1 mM Tris-HCl (pH 7.5). The sample was then applied to a double-stranded DNA–cellulose column that contained ~10 mg of DNA and that had been equilibrated to 0.2 M NaCl–1 mM Tris-HCl (pH 7.5). The material that failed to bind to the double-stranded DNA column was applied directly to a single-stranded DNA–agarose column that contained ~20 mg of DNA and that had been equilibrated to 0.2 M NaCl–1 mM Tris-HCl (pH 7.5). After washing with the same buffer, the proteins that bound to the single-stranded DNA–agarose column were eluted by applying solutions of higher NaCl concentrations. The fraction eluted by 0.3 M NaCl–1 mM Tris-HCl (pH 7.5) contained the majority of the cytosolic HMG-1. That protein was separated from a high molecular weight contaminant by chromatography on a 0.9 × 70 cm column of Sephadex G-100 in 0.1 M NaCl–1 mM Tris-HCl (pH 7.5).

The material precipitated by 40% saturated ammonium sulfate in the above scheme was judged to be devoid of HMG-1 by the failure to recover material with the electrophoretic properties of that protein when the precipitate was dissolved in water, exhaustively dialyzed against 0.2 M NaCl–1 mM Tris-HCl, and subjected to sequential chromatography on double-stranded and single-stranded DNA columns.

All steps in the isolation scheme after harvesting HTC cells were carried out at 0–4 °C.

Assessment of DNA Contamination in Cytosol Fraction. To a 100-mL exponential phase culture of HTC cells was added 100 μCi of [^{3}H]deoxythymidine. After 5 h the cells were harvested and the cytosol fraction was prepared as described above. Portions (0.5 mL) of the cell homogenate, the 4000*g* supernatant, and the 20000*g* supernatant were brought to 10% Cl_3AcOH–1% sodium pyrophosphate. The precipitates were transferred to Reeve Angel glass fiber filters and washed with 10% Cl_3AcOH–1% sodium pyrophosphate, 5% Cl_3AcOH–1% pyrophosphate, and absolute ethanol. The amount of radioactivity on each filter was determined by liquid scintillation counting in a nonaqueous scintillation fluid.

Antigens and Antibodies. The preparation of calf thymus HMG-1, the elicitation of specific antibodies, and their purification by affinity chromatography have been described previously (Bustin et al., 1978).

Immunofluorescence and Microphotography. HTC cells grown on microscope slides were fixed for 5 min with methanol at −20 °C and dipped in acetone at −20 °C. The air-dried slides were rehydrated with saline–phosphate buffer, precoated with bovine serum albumin, stained with 1:50 to 1:100 dilutions of either rhodamine-labeled affinity purified anti-HMG-1 antibodies or nonlabeled antibodies. When nonlabeled antibodies were used, their location was visualized with fluorescein-labeled protein A. For further detail, see Bustin & Neihart (1979).

Microcomplement Fixation. Quantitative microcomplement fixation assays (Wasserman & Levine, 1961) were used to determine the index of dissimilarity between HMG-1 proteins as described previously (Bustin et al., 1978; Romani et al., 1979).

Other Procedures. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed as described previously (Bidney & Reeck, 1978a). Acid–urea gel electrophoresis was carried out as described by Panyim & Chalkley (1969) except that we used slabs rather than cylindrical gels and the gels were polymerized from 20% acrylamide–0.3% bis(acrylamide). Protein concentrations were determined with the Coomassie blue binding assay of Bradford (1976) with ovalbumin as the standard. Amino acid analyses were carried out on a Durrum microbore kit analyzer (Dionex) equipped with a ninhydrin detection system and a Columbia Scientific Supergrator integrator.

Results

Immunofluorescence Studies. Immunofluorescence studies have revealed that while antibodies to histones specifically stain the nuclei of various cells, antibodies to protein HMG-1 stain both the nucleus and the cytoplasm (Bustin & Neihart, 1979). The photomicrographs presented in Figure 1 further emphasize the difference between the apparent cellular distributions of HMG-1 and that of histones. The figure depicts a preparation of human KD fibroblast cells in which the location of protein

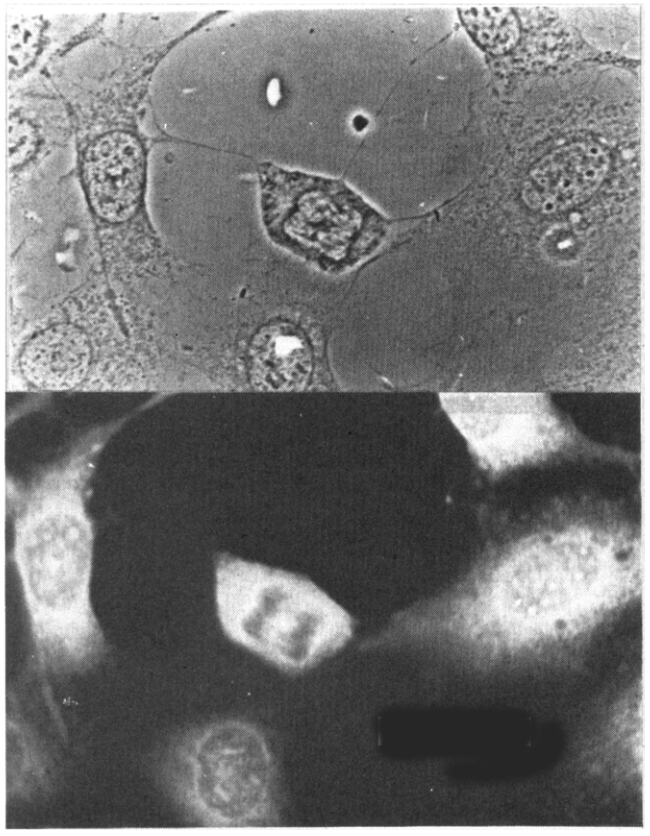

FIGURE 1: Depletion of HMG-1 antigenic determinants in chromosomes. The location of HMG-1 in human KD fibroblasts was visualized by indirect immunofluorescence as described by Bustin & Neihart (1979). Top panel: phase contrast micrograph. Bottom panel: corresponding fluorescence micrograph.

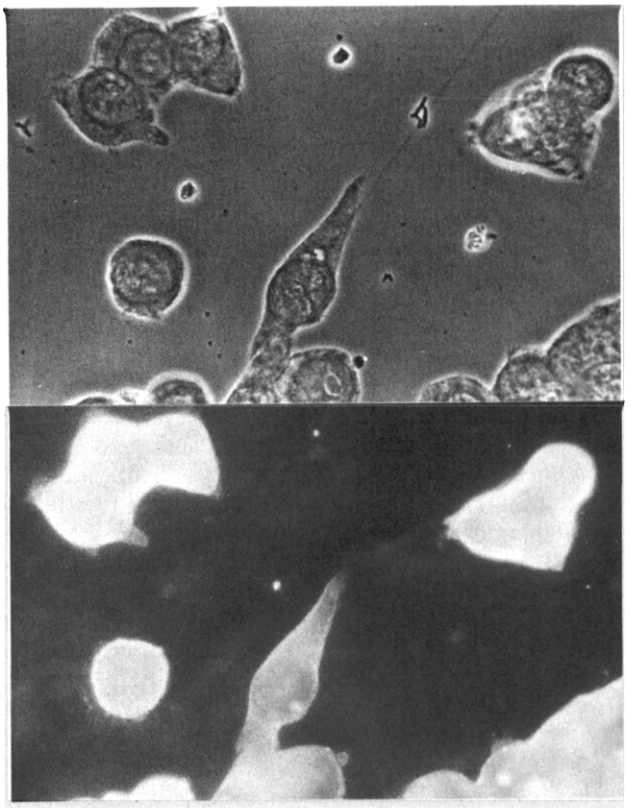

FIGURE 2: Localization of HMG-1 cross-reacting antigens in HTC cells. Top panel: phase contrast micrograph. Bottom panel: corresponding fluorescence micrograph. Anti-HMG-1 antibodies at 10 μg/mL and fluorescein-labeled protein A at 3 μg/mL were used as described under Materials and Methods.

HMG-1 was visualized by indirect immunofluorescence. The chromosomes visible by phase optics (cell in the center of the top and bottom panels) clearly display diminished fluorescence when viewed under fluorescence optics. A similar preparation stained with antibodies to histone H2B or H3 displays chromosomal fluorescence (data not shown). In the nonmetaphase cells in Figure 1, fluorescence is present in both nuclei and cytoplasm.

The photomicrographs presented in Figure 2 reveal that a similar situation exists in interphase HTC cells: the antibodies recognize components that are present both in the nucleus and in the cytoplasm. With these cells, the fluorescence intensity was somewhat weaker than that observed in rat liver TR-12 cells (Bustin & Neihart, 1979). Cells treated with nonimmune serum failed to display such fluorescence. We conclude that the staining pattern obtained with HTC cells is similar to that observed with other cells stained under similar conditions. That suggests that HMG-1 or immunologically cross-reacting material is present in both the nucleus and cytoplasm of HTC cells.

Purification and Electrophoretic Properties of Cytosolic HMG-1. At 0.2 M NaCl, the nuclear forms of the high molecular weight HMG proteins from several sources exhibit little or no affinity for double-stranded DNA; this permits a facile isolation by sequential chromatography on columns containing immobilized double-stranded DNA and single-stranded DNA (Bidney & Reeck, 1978b; Isackson et al., 1979). We therefore used that isolation scheme to attempt to purify HMG proteins from the cytosol fraction of HTC cells. Following the procedure described under Materials and Methods, we succeeded in purifying a cytosolic protein that chromatographed indistinguishably from nuclear HMG-1 on double-stranded and single-stranded DNA columns. The data described below show clearly that the protein is a cytosolic form of HMG-1.

$NaDodSO_4$–polyacrylamide gel analysis of the proteins present at several stages in the purification of cytosolic HMG-1 is shown in Figure 3. As in the case of nuclear HMG-1, the protein is not retained by the double-stranded DNA column at 0.2 M NaCl–1 mM Tris-HCl (pH 7.5) but is retained in single-stranded DNA chromatography carried out in the same solvent (tracks 1–3). Most of the HMG-1 was eluted from the single-stranded DNA column with 0.3 M NaCl–1 mM Tris-HCl (pH 7.5) (track 4). That fraction also contained a small amount of a high molecular weight contaminant that was removed from the HMG-1 by gel chromatography. The resulting preparation of HMG-1 is shown in Figure 3, track 7.

The purified protein had the same mobility in $NaDodSO_4$–polyacrylamide gel electrophoresis as nuclear HMG-1 from HTC cells (track 8). As shown in Figure 4, the cytosolic HMG-1 had the same mobility in acid–urea gel electrophoresis as nuclear HMG-1 from both HTC cells and calf thymus.

The yield of cytosolic HMG-1 was $\sim$1 μg/10^8 cells, which is comparable to the yield of nuclear HMG-1 obtained by Bidney & Reeck (1978b), who used chromatin as the starting material. The presence of HMG-1, at the level we have isolated it, in the cytosol fraction of HTC cells cannot be explained by contamination of cytosol by chromatin: using the approach described under Materials and Methods, we estimated that the amounts of contaminating DNA in the 4000g and the 20000g supernatants of the cell homogenate were only 4 and 1%, respectively, of the amount of DNA present in the cell homogenate itself.

A protein with the same mobility as HMG-2 was eluted

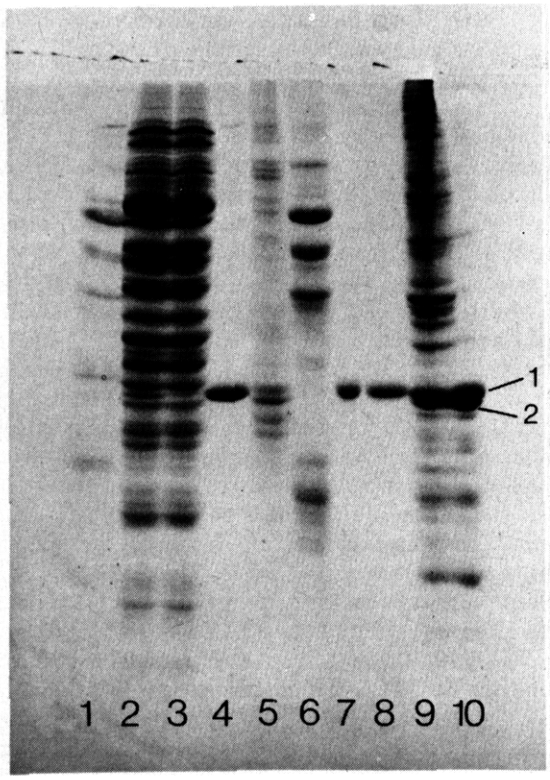

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gel analysis of the purification of cytosolic HMG-1 from HTC cells. Track 1: material retained by double-stranded DNA column and subsequently eluted with 2 M NaCl for analysis. Track 2: material that failed to bind to double-stranded DNA column at 0.2 M NaCl–1 mM Tris-HCl; this material was used as the sample for single-stranded DNA column. Track 3: flowthrough from single-stranded DNA column. Track 4: material eluted from single-stranded DNA column with 0.3 M NaCl–1 mM Tris-HCl. Track 5: material eluted from single-stranded DNA column with 0.5 M NaCl–1 mM Tris-HCl. Track 6: material eluted from single-stranded DNA column with 2 M NaCl–1 mM Tris-HCl. Track 7: cytosolic HMG-1 after chromatography on Sephadex G-100. Track 8: nuclear HMG-1 isolated from HTC cell chromatin (Bidney & Reeck, 1978b). Track 9: proteins extracted from HTC cell chromatin with 0.35 M NaCl. Track 10: HTC cell chromatin proteins extracted with 0.35 M NaCl and soluble in 2% Cl_3AcOH. The HMG-1 and HMG-2 bands are indicated.

from single-stranded DNA with 0.5 M NaCl–1 mM Tris-HCl (pH 7.5) (Figure 3, track 5). On the basis of both its elution behavior and its electrophoretic mobility, we assume that the protein is a cytosolic form of HMG-2.

Chemical and Immunochemical Properties of Nuclear HMG-1 and Cytosolic HMG-1. The immunological relatedness between HMG-1 from HTC cell nuclei and cytosol and HMG-1 from calf thymus was estimated by quantitative microcomplement fixation. For these studies we used previously characterized antibodies against calf thymus HMG-1. Complement fixation curves for the three proteins are shown in Figure 5. The data are plotted in Figure 6 in a manner that allows an estimation of the index of dissimilarity between the proteins (Champion et al., 1975). The ratio of the homologous serum dilution that gives 50% maximal complement fixation to the serum dilution for 50% maximal complement fixation for the HTC cell HMG-1 proteins gives the same index of dissimilarity (1.6) for the nuclear and cytosolic form of HTC cell HMG-1 with respect to that of calf thymus HMG-1. From this number one can estimate a percentage sequence difference of 4% for the HTC cell HMG-1's compared to calf thymus HMG-1.

Amino acid analyses of HMG-1 isolated from HTC cell nuclei and HTC cell cytosol are given in Table I. By this

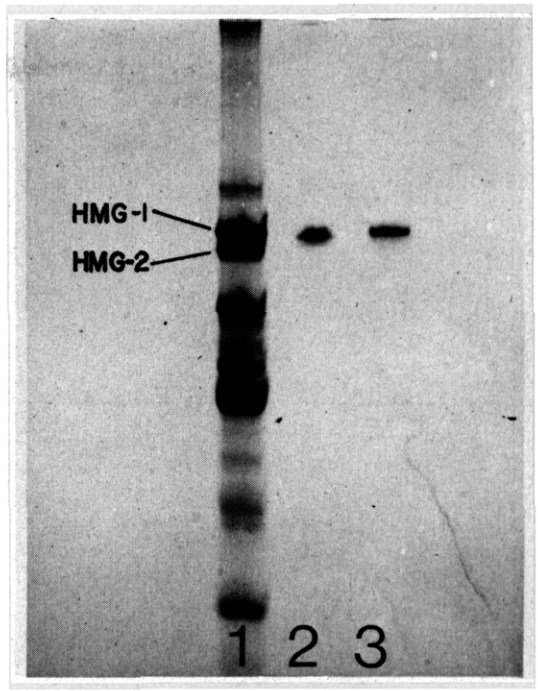

FIGURE 4: Acid–urea gel electrophoresis of HMG proteins. Track 1: calf thymus HMG protein preparation after the method of Goodwin et al. (1973). Track 2: nuclear HMG-1 from HTC cells. Track 3: cytosolic HMG-1 from HTC cells.

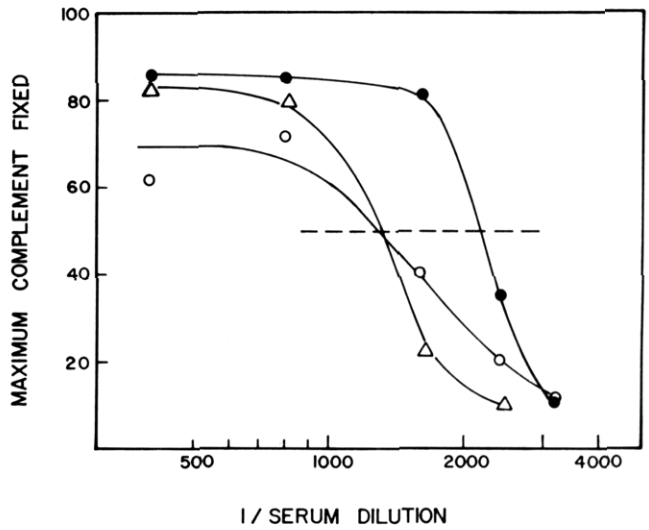

FIGURE 5: Complement fixation of antisera elicited by HMG-1 purified from calf thymus, with HMG-1 proteins purified from the nucleus and cytoplasm of HTC cells. The serum dilution for each set of curves is given in the upper right corner of each panel. (●) Reaction with immunogen (i.e., against calf thymus HMG-1); (○) reaction with nuclear HTC cell protein; (△) reaction with cytoplasmic HTC cell protein.

criterion the cytosolic and nuclear proteins appear very similar.

Discussion

By sequential chromatography on columns containing immobilized double-stranded DNA and single-stranded DNA, we have isolated a protein from the cytosol fraction of cultured rat hepatoma cells that is clearly an HMG protein. That conclusion is based on the protein's behavior on DNA columns and gel electrophoresis and on the protein's amino acid composition and immunochemical reactivity. Indeed, in all those properties the cytosolic HMG protein is very similar to nuclear HMG-1 isolated from the same line of cells. Taken together, our results suggest that the cytosolic form of HMG-1 in HTC

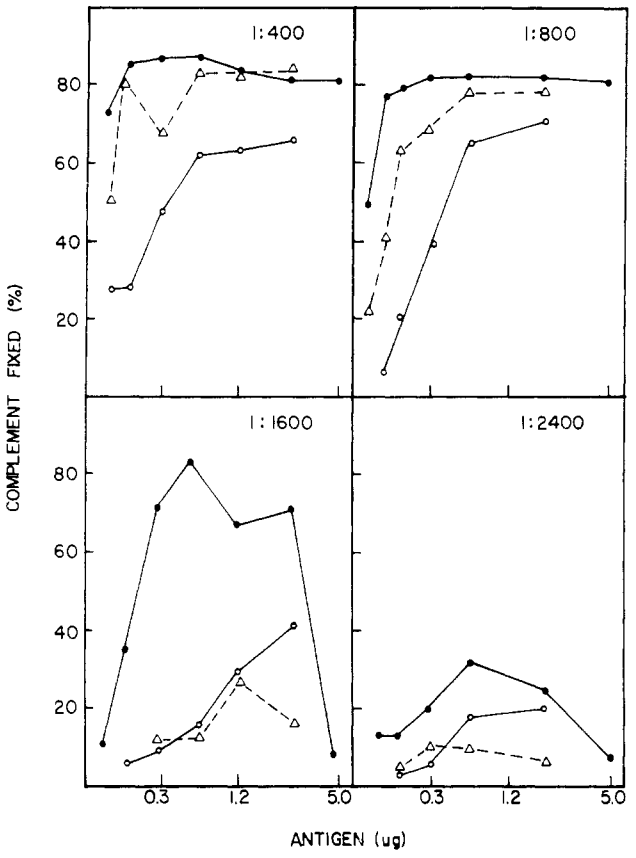

FIGURE 6: Determination of index of dissimilarity between HMG-1 proteins purified from calf thymus (●), nuclei of HTC cells (○), and cytoplasm of HTC cells (△). The maximal complement fixation obtained at various antisera dilutions was plotted against the reciprocal of that dilution.

Table I: Amino Acid Compositions of Nuclear and Cytosolic HMG-1's from HTC Cells[a]

amino acid	amino acid composition: cytosolic HMG-1[b]	nuclear HMG-1[c]
Asp	12	11
Thr	3.1	3.4
Ser	5.2	6.1
Glu	18	19
Pro	5.2	5.7
Gly	7.6	9.3
Ala	9.6	9.5
Val	3.2	2.4
Met	0.6	0
Ile	2.8	2.0
Leu	3.2	2.9
Tyr	0.8	1.4
Phe	3.5	3.6
Lys	17	17
His	2.6	2.5
Arg	4.5	3.7

[a] Values are in mole percent. [b] Average of two analysis after 24 h of hydrolysis. No corrections were made for partial destruction or incomplete release of any amino acid. [c] From Bidney & Reeck (1978b).

cells is identical with the nuclear form of HMG-1.

The occurrence of HMG-1 in the cytosol fraction of HTC cell homogenates cannot be attributed to chromatin contamination of our cytosol preparation, because the amount of DNA in the cytosol fraction was only 1% of the amount of DNA present in the cell homogenate, whereas the yield of HMG-1 from the cytosol was comparable (on a per cell basis) to the yield of HMG-1 from chromatin. Furthermore, we failed to observe any proteins with electrophoretic mobilities of histones bound to the double-stranded DNA column (Figure 3, track 1). We cannot, however, exclude the possibility that some of the cytosolic HMG-1 leaked out of nuclei when HTC cells were broken. Indeed, we cannot strictly exclude the possibility that the ability to isolate HMG-1 from nuclei or chromatin results from adsorption of the protein from the cytoplasm during or after the breakage of the cells.

There are, however, two studies with intact cells that suggest that HMG-1 occurs both in cytoplasm and in nucleus. In the first of those studies, Bustin & Neihart (1979) found that fluorescent-labeled antibodies against HMG-1 stained both the cytoplasm and nuclei of several types of mammalian cells. The intensities of fluorescence of cytoplasm and nuclei vary according to the cell type, fixation procedure, and phase of the cell life cycle. In most cells there is preferential fluorescence in the nucleus during the S phase (M. Bustin and N. K. Neihart, unpublished observations), but in the metaphase the chromosomes are totally devoid of fluorescence while the cytoplasm surrounding the chromosomes displays intense fluorescence (see Figure 1). In the second study, Rechsteiner & Kuehl (1979) followed the fate of radiolabeled HMG-1 after microinjection into mammalian cells. With HeLa cells, Rechsteiner & Kuehl (1979) found that 70% of the radioactivity was localized in nuclei. That suggests that, in HeLa cells, about two-thirds of HMG-1 is nuclear and one-third is cytoplasmic. Rechsteiner & Kuehl (1979) also found that in binucleated cells, HMG-1 was capable of transferring between nuclei, presumably through the cytoplasm.

It is clear, therefore, that the cellular distribution of HMG-1 differs from that of the histones, which are considered to be strictly chromosomal proteins. It is possible that HMG-1 shuttles between nucleus and cytoplasm. If, as Bidney & Reeck (1978b) have suggested, the high molecular weight HMG proteins are involved in DNA replication as helix-destabilizing proteins, a cell's requirement for the presence of the proteins in the nucleus would be greatest during the S phase.

The data presented in this paper demonstrate that HMG-1 can be isolated from the cytosol of a mammalian cell line. That does not exclude the possibility that some other material in the cytoplasm may also contribute to the fluorescence seen in Figures 1 and 2. Indeed, we believe that HMG-2, which is immunologically cross-reactive with HMG-1 (Bustin et al., 1978), also occurs in cytosol (see Results), and one of us (M.B.) is currently investigating the possibility that other immunologically cross-reactive cytosolic species exist.

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Third Component of Human Complement: Appearance of a Sulfhydryl Group following Chemical or Enzymatic Inactivation†

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ABSTRACT: Treatment of human C3 with hydroxylamine or hydrazine at physiological pH and ionic strength totally abrogates the intrinsic ability of this protein to sustain classical pathway induced hemolysis of sheep red blood cells. Concomitant with the loss of this function the appearance of a single sulfhydryl group can be followed by titration with the sulfhydryl-specific reagents *p*-(chloromercuri)benzoate, [1-^{14}C]iodoacetamide, 2,2′-dipyridyl disulfide, and 5,5′-dithiobis(2-nitrobenzoic acid). These reagents have also been used to follow the appearance of a free sulfhydryl group on conversion of C3 to C3b with bovine trypsin. Autoradiography of the electrophoretogram of separated α-, α'-, and β-polypeptide chains of inactivated, [1-^{14}C]carboxamidomethylated C3 samples has shown that the reactive sulfhydryl group is present in the α chain of C3 and in the α' chain of C3b, respectively. Digestion of the radiolabeled protein with porcine elastase has localized this sulfhydryl group to a 28 000-dalton fragment of the α chain with immunochemical and functional reactivities of the C3d domain. Autoradiographic analysis of a hydrolysate prepared from radioalkylated C3 and subjected to high-voltage paper electrophoresis has shown the labeled amino acid to be [1-^{14}C]-*S*-(carboxymethyl)cysteine. The susceptibility of native C3 to rapid and irreversible inactivation by nitrogen nucleophiles with the parallel appearance of a cysteinyl residue may indicate the presence of an internal thiol ester. The relationship of the proposed thiol ester to the ability of nascent C3b to acylate cell surface components and carbohydrate polymers is discussed within the context of a transesterification reaction.

The third component of human complement, C3, contributes importantly to immune surveillance and immune response pathways. The activation of C3 and subsequent binding of nascent C3b to a foreign cell or particle surface strengthen interactions with cellular elements operative in host defense. Complement (C) receptors specific for stable binding sites present on C3b, C3bi, and C3d have been detected on peripheral blood cells of many vertebrate species and include the neutrophil, eosinophil, monocyte, B-lymphocyte, and primate erythrocyte. Three distinct receptors have been identified and shown to bind to different regions of the C3 molecule (Gigli & Nelson, 1968; Ross & Polley, 1975; Ross & Rabellino, 1979). The importance of this class of protein–protein receptor interaction to defense mechanisms is highlighted by two sets of observations: (1) the studies of Alper et al. (1972) of a patient with inherited C3 deficiency indicated that her serum could not sustain opsonization of bacteria and she was, therefore, subject to severe and recurrent bacterial infections and (2) the studies of Ehlenberger & Nussenzweig (1977) on the respective roles of IgG and C3 for phagocytosis of sheep erythrocytes by monolayers of human monocytes and neutrophils indicated separate but synergistic roles for these two opsonins. The primary role of C receptors on these cells was to facilitate contact with the opsonized erythrocyte, and the role of the Fc receptor was primarily one of triggering the ingestive processes. Studies by Lewis et al. (1977) of immune response pathways have shown an obiligatory C3 requirement for complement receptor positive β-lymphocytes to respond in vivo or in vitro to T-cell-dependent antigens, suggesting that C3 receptors may be required for T-cell–B-cell cooperative responses, perhaps at the macrophage level. Therefore, in addition to their specific involvement in immune clearance pathways, these receptors may well be required for the modulation of the B-lymphocyte response to T-dependent antigens.

In consideration of the positive control exerted by C3 on bacterial pathogens, it is important to elucidate the mechanism by which nascent C3b binds to cell surface structures. The association of C3b with components present on all cell types (Götze & Müller-Eberhard, 1970; Müller-Eberhard, 1975) and many carbohydrate polymers including zymosan (Nicholson et al., 1974), dextran (Arnaout et al., 1979), and agarose (Goldstein et al., 1976; Capel et al., 1978) following "classical" or "alternative" pathway activation has been de-

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