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Interaction of chromosomal proteins with BrdU substituted DNA as determined by chromatin-DNA competition

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ABSTRACT

Chromatin-DNA competition has been utilized to examine the general nature of chromosomal proteins interacting more strongly with BrdU substituted DNA. When chromatin is incubated with an excess of purified DNA, a portion of the chromosomal proteins will exchange to the purified DNA. These two complexes can then be separated on Metrizamide gradients due to their differing protein/DNA ratios. Using this technique we observe that most nonhistone chromosomal proteins will exchange to a competitor DNA, the extent of exchange being directly dependent upon the competitor DNA being present in excess. While essentially the same proteins will migrate to either unsubstituted or BrdU substituted DNA, the substituted DNA is found to be a quantitatively better competitor and its effectiveness as a competitor is directly related to the level of BrdU substitution.

INTRODUCTION

The observation that the thymidine analog, 5-bromodeoxyuridine (BrdU), will alter the expression of specific genetic loci in animal cells (1-4) is frequently considered to be related to the demonstration of Lin and Riggs that both structural and regulatory proteins will bind more tightly to substituted DNA. This phenomenon has been most extensively investigated by the filter binding technique with the lac repressor (5), CAP protein (6), and animal cell histones (7). In order to further investigate the possibility that BrdU acts on gene expression through an altered binding of regulatory proteins to DNA it is important to establish whether or not this tighter binding phenomenon extends to other chromosomal proteins as well. To that end we have developed a technique, termed chromatin-DNA competition, in order to ascertain the global nature of chromosomal proteins interacting more strongly with BrdU substituted DNA.

The technique of chromatin-DNA competition relies upon the nonionic density gradient medium, Metrizamide. When chromatin and a purified DNA are incubated together under appropriate conditions, a population of chromatin associated proteins will migrate to the purified DNA if presented in excess. The original chromatin and the new DNA-protein complexes can then be separated on

Metrizamide gradients due to their differing protein/DNA ratio. We have applied this approach to determine if BrdU substituted DNA is a stronger competitor, and by analysis of the exchanged proteins, whether the stronger association of proteins to substituted DNA extends to most chromosomal proteins.

MATERIALS AND METHODS

Cell Culture Labeling: Growth and maintenance of the cell lines used here has been described (8,9). Briefly, 3460 is a Syrian hamster melanoma line grown in the absence of BrdU. HAB is a cell line derived from 3460 which is maintained in hypoxanthine-aminopterin-BrdU, resulting in the DNA being 100% substituted with BrdU. To achieve various levels of BrdU substitution in DNA, 3460 cells were grown for 8 days in BrdU ranging from 10^{-6} M to 10^{-5} M. DNA was isolated (8), treated with S1 nuclease, and the extent of substitution determined by analytical CsCl centrifugation as described by Luk and Bick (10).

3460 cell chromatin was double-labeled by growth for 72 hrs in 7 µc/ml ^{35}S-methionine and 1.2 µc/ml ^{3}H-TdR. HAB cell chromatin was similarly labeled except that 3 µc/ml ^{3}H-BrdU was used for DNA labeling. In some cases, cells were grown in 28 µc/ml ^{3}H-leucine for labeling of proteins alone, or in ^{3}H-TdR (3460 cells) and ^{3}H-BrdU (HAB cells) for labeling of only DNA. Due to the increased photosensitivity of cells and DNA substituted with BrdU, cell growth and all subsequent manipulations were carried out in an environment where all light below 550 nm was filtered out.

Chromatin Isolation: Cells were scraped from the culture dishes in 10 mM Tris-Cl, pH 7.4, 2 mM $MgCl_2$, 7 mM 2-mercaptoethanol, and 0.5 mM phenylmethanesulfonyl fluoride (PMSF). The cell suspension was Dounce homogenized and spun at 300 xg for 5 min. The nuclear pellet was resuspended in 0.32 M sucrose, 3 mM $MgCl_2$, 0.1% Triton X-100 and Dounce homogenized. This suspension was layered over 1.7 M sucrose, 1 mM $MgCl_2$, and spun at 22K rpm for 75 min in the SW-27 rotor. The nuclear pellet was recovered and lysed by a series of washes in decreasing ionic strength (20 mM to 5 mM Tris, containing 0.5 mM PMSF and 0.1% Triton). Chromatin suspensions were stored frozen at -70°C.

Chromatin-DNA Competition: The incubation mixture consisted of: 10 mM Tris, pH 7.4, 1 mM EDTA, 50 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM PMSF, 0.1% Triton X-100, 20-50 µg/ml chromatin DNA, and the indicated excess of purified competitor DNA. Incubation was for 2 hrs at 37°C, after which the mixture was gently layered over 5 ml of 44% (w/v) Metrizamide in 10 mM Tris, pH 7.4, 1 mM EDTA, 50 mM NaCl, and 0.1% Triton X-100. Gradients were spun at 35 K rpm for 17-18 hrs in the Ti 50 rotor. Tubes were punctured and 13 drop fractions

collected from below. An equal volume of 5 M NaCl was added to each fraction to disperse chromatin clumps and 10 µl aliquots were taken for counting in 10 ml ACS (Amersham/Searle). Data were calculated and plotted by computer. All gradients received 3 x 10^4 to 2 x 10^5 cpm.

SDS-gel Electrophoresis: The indicated fractions were pooled, dialyzed overnight against 0.5 mM PMSF, lyophilized, resuspended in 50 µl sample buffer, and applied to a 15% SDS-polyacrylamide slab gel (19 cm x 14 cm). The Tris-glycine gel system of Laemmli (11), as modified for slab gels by Anderson et al. (12), was used. Gels were run for 18 hrs at 10 mA and dried under vacuum for autoradiography. Gels were prepared for fluorography as described by Bonner and Laskey (13) and exposed to presensitized SB-54 film (Kodak) at -70°C.

Materials: ^{35}S-methionine (425 Ci/mmole), ^{3}H-TdR (48 Ci/mmole), and ^{3}H-BrdU (4.4 Ci/mmole) were purchased from Amersham/Searle. ^{3}H-leucine (58 Ci/mmole) was obtained from New England Nuclear. Metrizamide [2-(3-acetamido-5-N-methylacetamido-2,4,6-triodobenzamido)-2-deoxy-D-glucose] was purchased from Accurate Chemical and Scientific Corporation (Hicksville, New York).

RESULTS

When double-labeled chromatin from 3460 cells is spun in a 44% Metrizamide gradient for 17-18 hrs, it bands as a sharp homogenous peak (ρ = 1.20) containing the vast majority of both DNA and protein radioactivity at a coincident position (Fig. 1A; note aggregated clump in Fig. 3). Similar gradients containing only purified DNA from either 3460 cells (Fig. 1B), or HAB cells (Fig. 1C) result in the DNA banding at a much migher position in the gradient (ρ = 1.18; see Fig. 4A for typical density range under these conditions). It is interesting to note that 100% BrdU substituted DNA bands at essentially the same position as unsubstituted DNA, unlike the substantial density differences observed in cesium salts.

Given the large difference in banding position of chromatin and purified DNA under these centrifugation conditions, resulting from the property of Metrizamide to fractionate on the basis of protein/DNA ratios (14,15), it seemed likely that any new protein-DNA complexes formed under our conditions for chromatin-DNA competition (see Materials and Methods) could be separated by such gradients. When ^{35}S-methionine labeled 3460 cell chromatin is competed with a 1, 4, or 16-fold excess of unlabeled HAB cell DNA, there is an increasingly larger fraction of ^{35}S-labeled proteins appearing coincident with the competitor DNA (Fig. 2). When similar competition reactions are carried out using double-labeled chromatin, there is no increase in the ^{3}H-DNA radio-

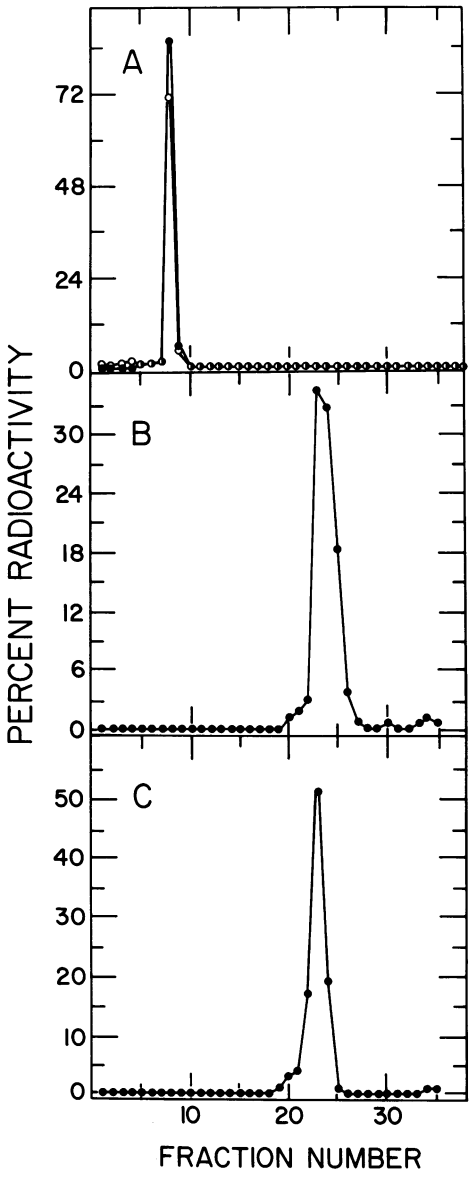

Fig. 1. Banding of chromatin and DNA in Metrizamide. Samples were layered on 44% Metrizamide and spun as described in Materials & Methods. (A) ^{35}S-methionine + ^{3}H-TdR labeled 3460 chromatin; (B) ^{3}H-TdR 3460 DNA; (C) ^{3}H-BrdU HAB DNA. Density increases to the left. ^{35}S - (O); ^{3}H - (●).

activity appearing coincident with the light fraction of ^{35}S-protein, excluding the possibility that these proteins are retaining their original association with the chromatin DNA but at a lower protein/DNA ratio (data not shown). These results suggest that chromosomal proteins from the 3460 cell chromatin are migrating from their original association and forming new complexes with

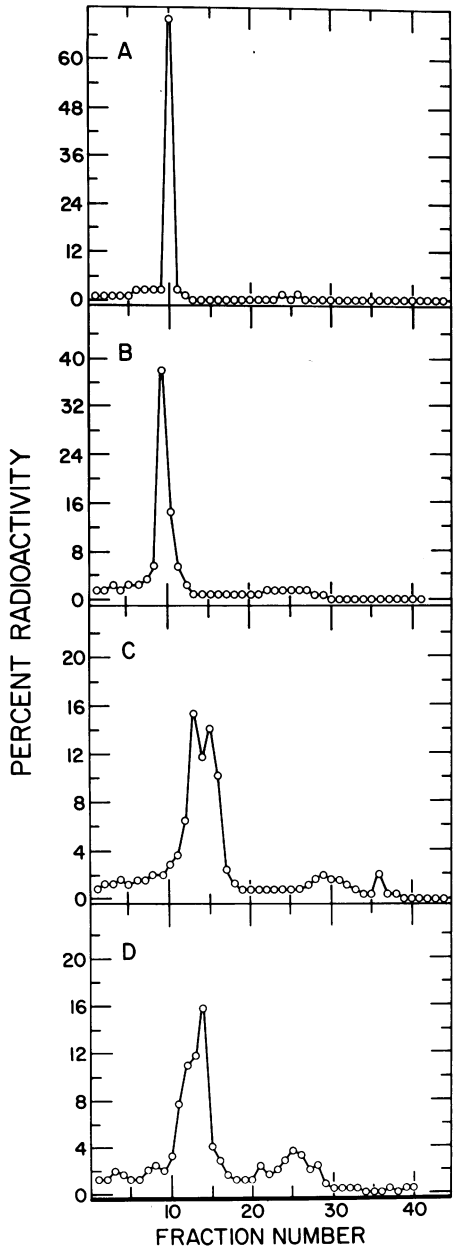

Fig. 2. 3460 cell chromatin competed by excess HAB DNA. ^{3}H-leucine (O) labeled 3460 cell chromatin was incubated with no DNA (A), or a 1-fold (B), 4-fold (C), or 16-fold (D) excess of unlabeled HAB cell DNA. Samples were then run on 44% Metrizamide gradients.

the purified competitor DNA. It is also apparent that the amount of exchange depends upon to what extent the competitor DNA is present in excess. When ethidium bromide (20 µg/ml) is added to the Metrizamide solution, the developed gradient can be observed under ultraviolet light. The chromatin is observed as a clump low in tube, whereas the competitor DNA fluoresces brightly at its characteristic position (Fig. 3).

That these putative new protein-DNA complexes actually represent a true association of proteins with the competitor DNA is demonstrated in Fig. 4. 3460 chromatin was first competed with a 4-fold excess of ^{3}H-HAB cell DNA, wherein a small fraction of the ^{35}S-labeled proteins now band coincident with ^{3}H-DNA (Fig. 4A). When these fractions were pooled and rerun on a similar gradient, the ^{35}S- and ^{3}H-radioactivity were again found coincident (Fig. 4B). However, when this pooled complex was treated with 2 M NaCl prior to rebanding (Fig. 4C), there is a broader distribution of ^{35}S-labeled proteins throughout

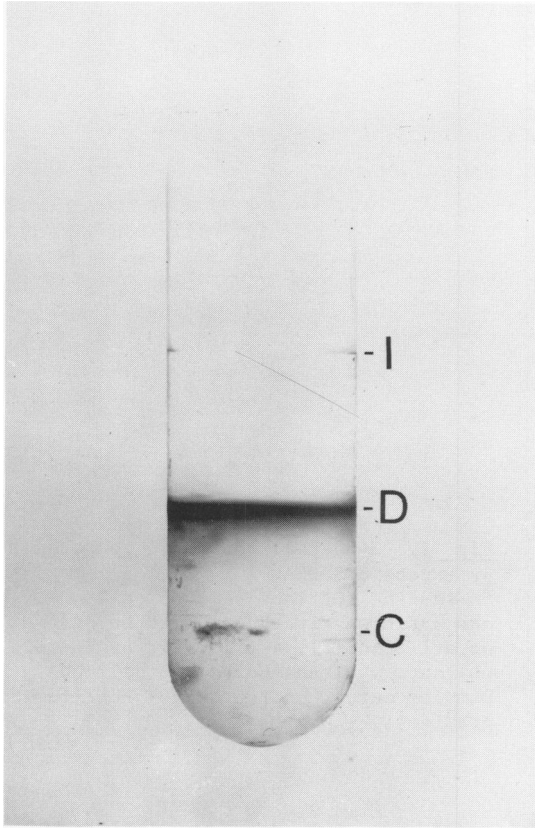

Fig. 3. Ethidium bromide stained Metrizamide gradient. A replicate gradient of Fig. 2C received 20 µg/ml ethidium bromide prior to loading the sample. The developed gradient was photographed under ultraviolet light. I, interface of gradient and oil overlay; D, competitor DNA; C, chromatin.

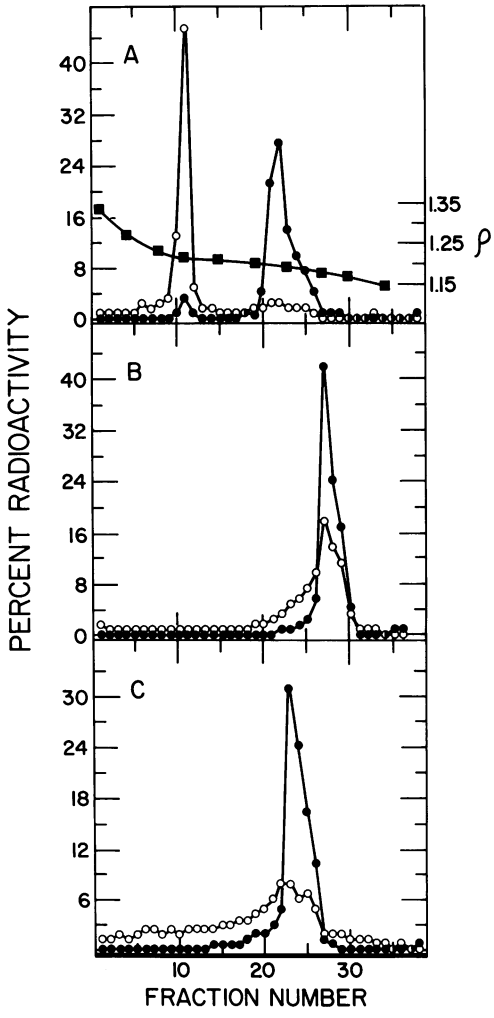

Fig. 4. Rebanding of DNA-protein complex. ^{35}S-methionine labeled 3460 chromatin was competed by a 4-fold excess of ^{3}H-HAB cell DNA (A). Fractions 20-26 were pooled and either rerun directly (B), or made 2 M with NaCl (C) before rerunning on a 44% Metrizamide gradient. The first gradient did not receive 5 M NaCl during sampling, as was usually the case, in order to preserve protein-DNA associations. ^{35}S·protein - (O); ^{3}H-DNA - (●); density in g/cm^3 - (■).

the gradient, suggesting that a portion of them have become dissociated from DNA under these moderate salt conditions.

Inasmuch as these data indicate that chromosomal proteins can be competed by an exogenous DNA, and these new complexes separated, it was of interest to determine the relative effectiveness of unsubstituted versus BrdU substituted DNA as competitors. Figure 5 shows a series of gradients in which 3460 cell chromosomal proteins are competed by DNAs ranging from 0% to 100% substitution. In all cases, the competitor DNA is added in an 8-fold excess. While purified 3460 cell DNA (unsubstituted) will attract a small fraction of protein label under these conditions, it is generally observed that a quan-

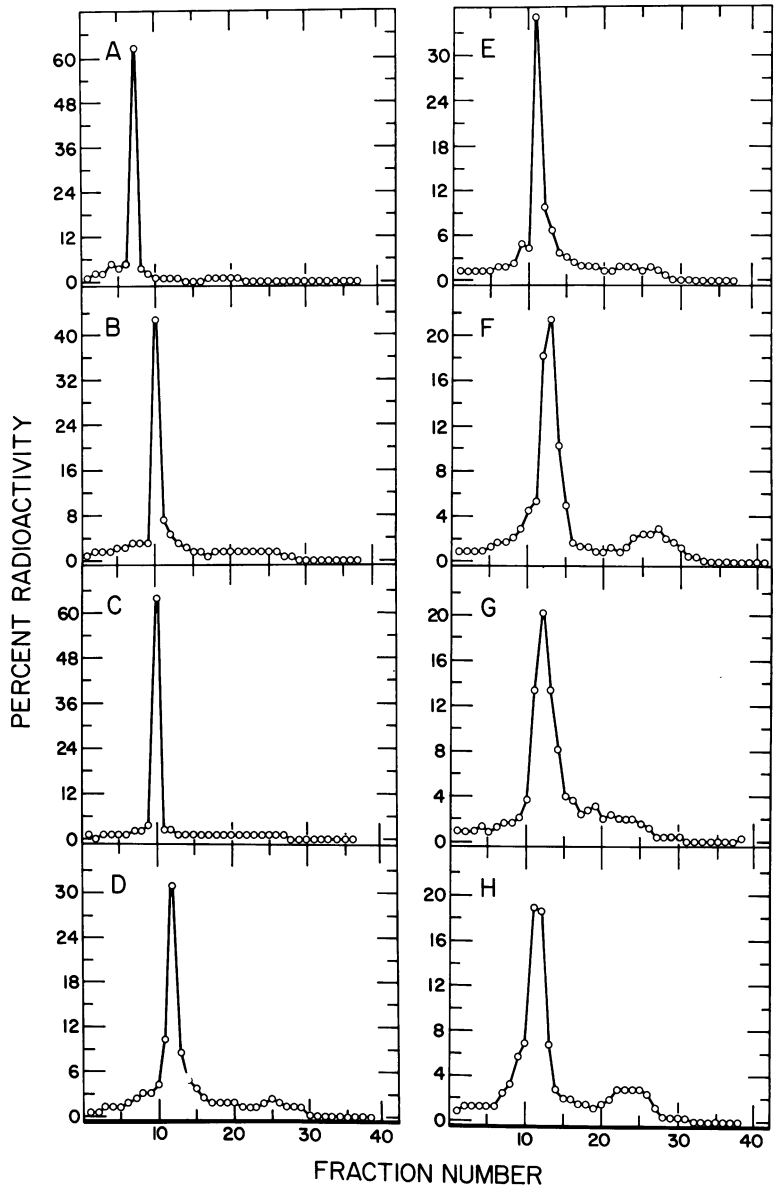

Fig. 5. 3460 cell chromatin competed with various BrdU substituted DNAs. ^{3}H-leucine (O) labeled 3460 cell chromatin was competed with an 8-fold excess of unlabeled DNA under standard conditions and then run on 44% Metrizamide. (A) no competitor; (B) 0%; (C) 6%; (D) 18%; (E) 46%; (F) 57%; (G) 82%; (H) 100% BrdU-substituted DNA.

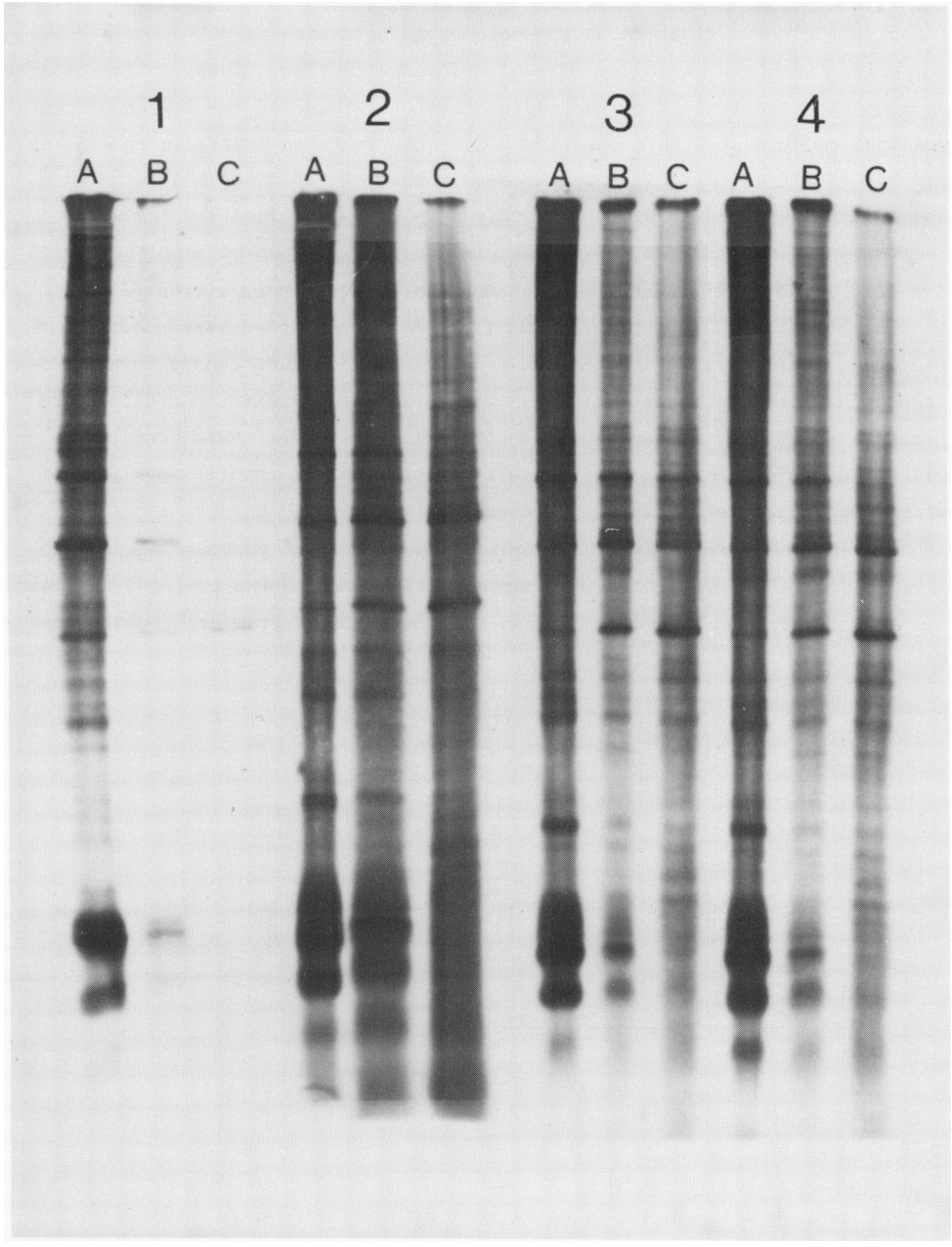

Fig. 6. SDS-Polyacrylamide gel pattern of competed proteins.
Samples were taken from the indicated gradients of Fig. 5.
1a,b,c: fractions 6-9, 10-14, 16-22, respectively of gradient A;
2a,b,c: fractions 8-12, 13-17, 18-28, respectively of gradient B;
3a,b,c: fractions 9-16, 17-21, 23-32, respectively of gradient F;
4a,b,c: fractions 7-14, 15-19, 20-32, respectively of gradient H.

titatively greater fraction of the chromosomal proteins is attracted to the competitor with increasing amounts of BrdU substitution. Thus, in the absence of competitor, only 4-5% of the radioactivity is found in the region of the gradient coincident with a purified DNA. This fraction increases to 13%, 19%, and 24% ($\pm$ $\sim$1% in duplicate experiments) when the competitor DNA is 0%, 57%, and 100% substituted by BrdU, respectively. When proteins from these new complexes are analyzed by SDS-polyacrylamide gels, it can be seen that there are few, if any, differences in the population of proteins attracted to unsubstituted versus substituted DNA (Fig. 6). With the exception of the histones, nearly all of the chromatin-associated proteins can be found associated with the competitor DNA. It, therefore, appears that chromosomal proteins capable of exchanging to a competitor DNA under these conditions will do so to either DNA, yet the substituted DNAs are quantitatively more effective in attracting these proteins.

To further investigate the tighter association of chromosomal proteins with BrdU substituted DNA, we have essentially reversed the protocol and asked how effectively unsubstituted DNA can compete proteins from BrdU substituted

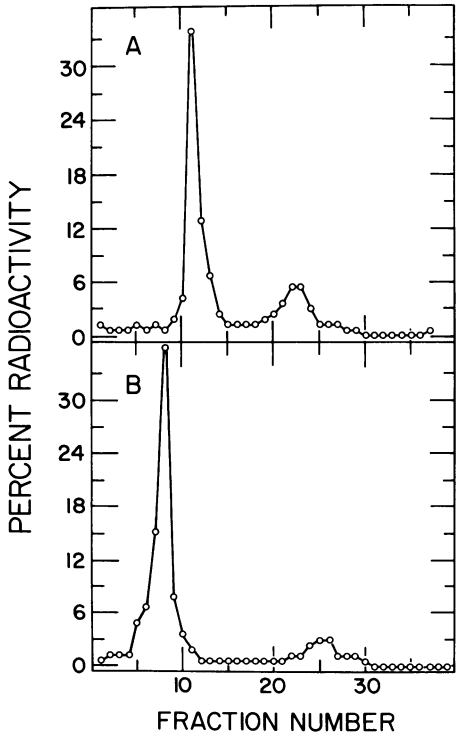

Fig. 7. HAB and 3460 cell chromatin competed by 3460 DNA. ^{35}S-methionine (O) labeled chromatin from either 3460 cells (A) or HAB cells (B) was competed by a 16-fold excess of unlabeled 3460 cell DNA.

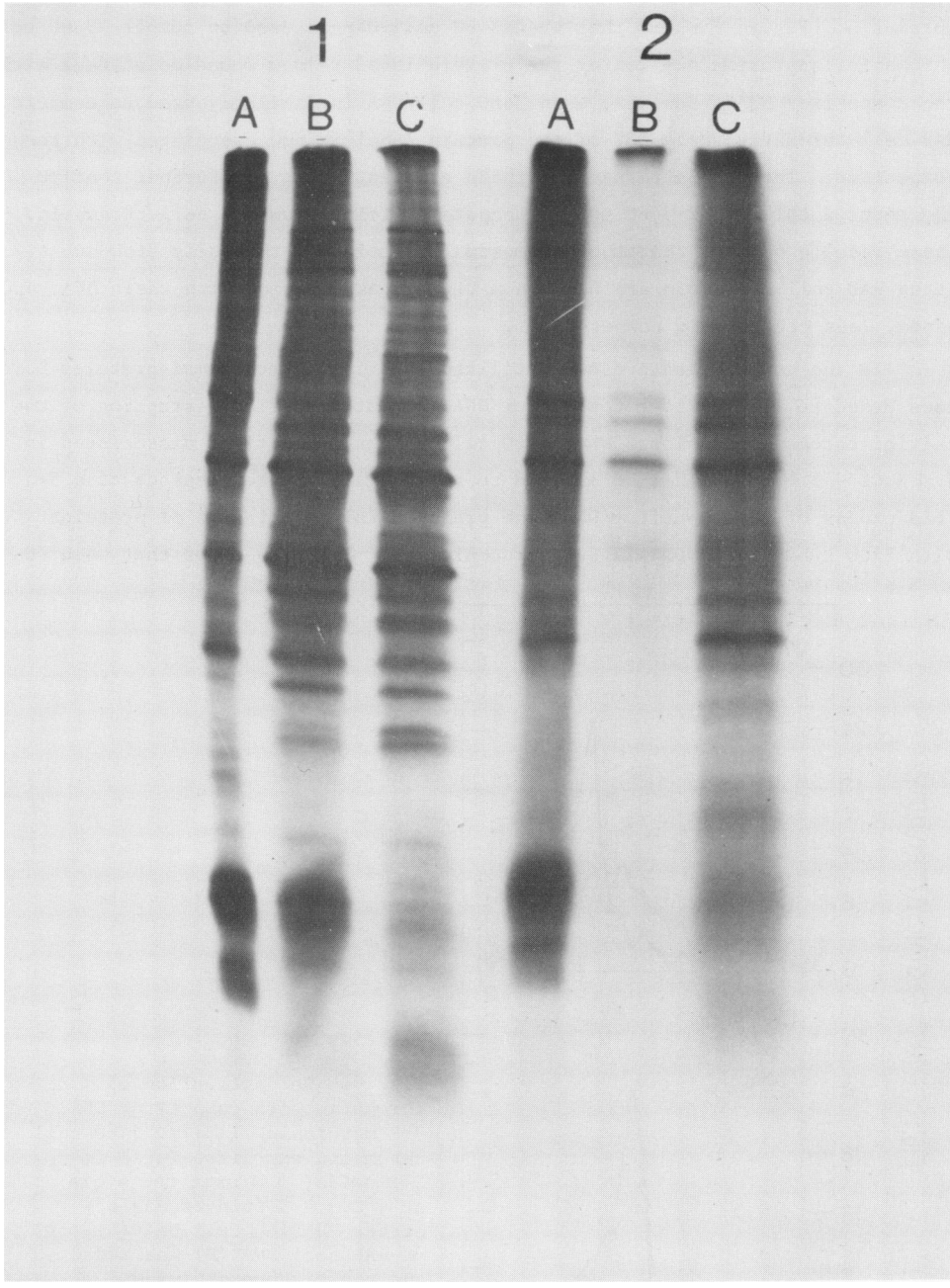

Fig. 8. SDS-polyacrylamide gel pattern of proteins competed from 3460 cell chromatin or HAB cell chromatin.
Samples were taken from Fig. 7A (fractions 9-14, 15-19, 20-29 are 1a, b, c, respectively) or Fig. 7B (fractions 4-12, 14-21, 23-32 are 2a, b, c, respectively).

chromatin. Figure 7 shows that when 3460 cell DNA is used to compete 3460 cell chromatin, approximately 26% of the protein label is now found coincident with the competitor DNA. However, when 3460 cell DNA is similarly used to compete HAB cell chromatin, only 13% of the protein label is now associated with the competitor. The gel patterns from these gradients (Fig. 8) further confirm the reduced ability of 3460 DNA to compete proteins from HAB cell chromatin. These results suggest that the chromosomal proteins of the fully BrdU-substituted HAB cell chromatin are held in a tighter association with their DNA, and hence, less effectively competed off.

In the competition protocol utilized thus far, chromosomal proteins have been asked to migrate to a competitor DNA without any prior disruption of the initial chromatin complex. When 3460 cell chromatin is first dissociated in 2 M NaCl - 5 M urea and then allowed to reassociate in the presence of a 5-fold excess of ^{3}H-competitor DNA, the preferential association of proteins with BrdU substituted DNA is further confirmed. Figure 9 shows that when ^{3}H-

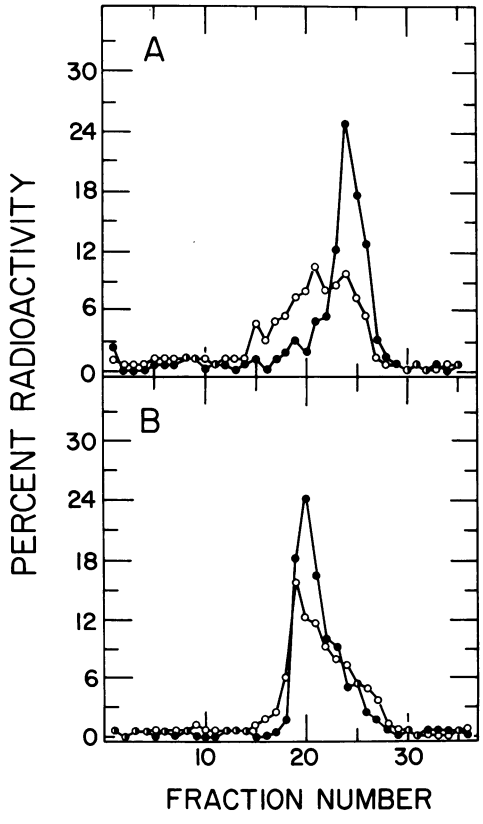

Fig. 9. Reconstitution of chromatin with competitor DNA. ^{35}S-methionine labeled 3460 cell chromatin was dissociated in 2 M NaCl - 5 M urea for 4 hrs at 4°C. A 5-fold excess of either ^{3}H-3460 cell DNA (A), or ^{3}H-HAB cell DNA (B) was added and the mixture dialyzed overnight against 10 mM sodium phosphate. The mixtures were loaded onto 44% Metrizamide gradients. ^{35}S - (O); ^{3}H - (●).

3460 cell DNA is used as the competitor, only about 50% of the proteins are coincident with the major peak of competitor DNA. When reassociation takes place in the presence of HAB cell DNA, the chromosomal proteins are nearly exactly coincident with the competitor DNA. A control gradient in which the dissociated chromatin was simply loaded onto Metrizamide, without dialysis against low salt, showed that 50-55% of the protein label bands in the lower 10 fractions of the gradient, with the remainder being randomly distributed throughout the lighter fractions (data not shown).

DISCUSSION

The technique of chromatin-DNA competition has allowed us to examine the global nature of chromosomal proteins interacting more strongly with BrdU substituted DNA. Under the exchange conditions described here the majority of non-histone chromosomal proteins will migrate to a competitor DNA. The extent of migration is dependent upon the competitor DNA being present in excess as demonstrated by the quantitatively increasing fraction of proteins becoming associated with the competitor as it is increased in amount. It should be noted, however, that there may be some limit to the amount of exchange which can occur under these conditions. Figure 2 shows a quantitatively greater fraction of protein attracted to HAB cell DNA as it increased up to a 16-fold excess, resulting in ∿26% of the protein label being exchanged. Similarly, when 16x 3460 DNA is used, approximately 26% of the label is also exchanged (Fig. 7A). We do not yet know if there may be some limitation to the allowable exchange under these conditions; indeed, the mechanism(s) involved in the exchange phenomenon itself remains obscure.

We have demonstrated that while both unsubstituted and BrdU-substituted DNA can attract essentially the same proteins under these conditions, the substituted DNA is quantitatively a better competitor. These results show that within the limits of resolution, chromosomal proteins will interact more strongly with substituted DNA. While the histone proteins do not show significant exchange under these relatively mild conditions, Lin *et al.* (7) have shown that they do bind more tightly to substituted λ DNA by the filter binding technique and we have confirmed that observation using their method with the 3460 and HAB cell DNA (T. Fasy and M. Bick, unpublished observation).

These results strengthen the possibility that BrdU may alter the expression of specific genetic loci in animal cells through an altered binding of regulatory proteins. It is interesting to note that two systems we have studied in detail, namely the inhibition of erythroid differentiation in Friend leukemia cells by BrdU (16), and the induction of deoxycytidine dea-

minase by BrdU in a hamster cell line (17), both show a nearly linear response with respect to the extent of inhibition (or induction) versus the degree of BrdU substitution in DNA. These biological phenomena correlate well with the observation reported here that the extent of tighter binding is related to the extent of substitution.

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