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Enhanced phosphorylation of high-mobility-group proteins in nuclease-sensitive mononucleosomes from butyrate-treated HeLa cells

(hyperphosphorylation/transcriptionally active chromatin/sodium butyrate)

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ABSTRACT **The protein composition of nucleosome fractions differing in their sensitivity to micrococcal nuclease and derived from butyrate-treated or untreated HeLa cells has been compared. Most of the high-mobility-group-14 (HMG-14) and HMG-17 content of HeLa cell chromatin is associated with those nucleosomes that are preferentially sensitive to micrococcal nuclease. Furthermore, electrophoresis of these two HMG proteins from the transcriptionally active chromatin fraction MN_1 of butyrate-treated cells resolves them into a series of bands. The multiple band pattern of HMG-14 and -17 from butyrate-treated cells results from hyperphosphorylation rather than hyperacetylation. Phosphorylation of these two small nonhistone proteins may play some role in the modulation of the structure of transcriptionally active chromatin.**

The addition of sodium butyrate to HeLa cells induces a number of morphological and biochemical changes, including increases in the rates of synthesis of various enzymes (1). During this induction period, the histones—particularly H3, H4, and H2B—become highly acetylated (2, 3) due to an inhibition of the histone deacetylase (4–6). The highly acetylated chromatin from butyrate-treated cells is more susceptible to digestion by DNase I (2, 7) and there are changes in the accessibility of particular DNA sites within the nucleosome core particle to attack by DNase I (8). Although the details of the mechanisms by which butyrate causes these alterations are largely unknown, it is likely that increased levels of histone acetylation may engender changes in the patterns of gene expression in HeLa cells. Nevertheless, many of the gross physicochemical properties of core particles from butyrate-treated and control HeLa cells remain similar (8).

As an initial approach to understanding the action of butyrate at the molecular level, I have compared the composition of acid-soluble proteins of micrococcal nuclease-sensitive (transcriptionally active) and -resistant (transcriptionally inactive) chromatin regions derived from butyrate-treated and control HeLa cells. The results show that butyrate promotes hyperphosphorylation of the high-mobility-group proteins HMG-14 and HMG-17. Because these two proteins are found in increased amounts in nuclease-sensitive chromatin (9), this increase in the phosphorylation of HMG-14 and -17 may either cause or be the consequence of alterations in the patterns of gene expression promoted by sodium butyrate.

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MATERIALS AND METHODS

Cell Cultures. HeLa cells, line S3, were grown in monolayers, either in T-150 culture flasks or in roller bottles, at 37°C in minimal essential medium (Flow Laboratories, Rockville, MD) supplemented with 7% bovine calf serum (KC Biologicals) and penicillin (100 units/ml) and streptomycin (100 μg/ml) (Sigma). In experiments involving sodium butyrate, this compound was added to the cultures at a final concentration of 5 mM about 20 hr prior to harvesting of the cells.

Preparation of Nuclei. HeLa cell nuclei were prepared as described by Milcarek *et al.* (10). In brief, cells that had formed a confluent monolayer were scraped from the bottles with the aid of a rubber policeman and recovered by centrifugation at 2000 rpm for 10 min in a Sorvall GSA rotor. The cell pellets were then resuspended in 10 mM NaCl/40 mM Tris·HCl, pH 8.3/1.5 mM $MgCl_2$ to which 12.5 μg of polyvinyl sulfate and 17.5 μg of spermine had been added just prior to use. Cells were homogenized gently in a Dounce homogenizer and then lysed by the addition of Nonidet P-40 at a final concentration of 0.5% for 2 min at 4°C. Nuclei were pelleted by centrifugation at 4000 rpm for 10 min in a Sorvall SS-34 rotor and washed once with 10 mM NaCl/10 mM Tris·HCl, pH 7.5/3 mM $MgCl_2$ (RSB buffer).

Micrococcal Nuclease Digestions. Nuclei derived from control or butyrate-treated HeLa cells were fractionated into transcriptionally active and inactive moieties by the method of Levy-Wilson *et al.* (11–13). Samples of intact nuclei were washed twice by gentle homogenization with RSB buffer containing 1 mM $CaCl_2$, followed by centrifugation for 5 min at 5000 rpm in a Sorvall HB-4 rotor at 0°C. The washed nuclei were gently resuspended in RSB/Ca^{2+} buffer at a concentration of about 100 A_{260}/ml. Micrococcal nuclease (Worthington) was added (1 unit/A_{260}) and digestion proceeded for 10 min at 37°C. The reactions were stopped by chilling the tubes on ice followed by centrifugation for 5 min at 5000 rpm (HB-4 rotor); the supernatant was designated S_1. The precipitate was homogenized in 1 mM EDTA (pH 7.5) for 20 min at 4°C, followed by centrifugation for 20 min at 10,000 rpm, to yield a second supernatant, S_2, and a pellet, P. S_2 was further fractionated by the addition of 0.1 M NaCl as described (12), to yield fractions MN_1 and MN_2.

Extraction of Acid-Soluble Proteins and Gel Electrophoresis. Acid-soluble proteins were obtained from either intact

Abbreviation: HMG, high mobility group.

nuclei or chromatin fractions as described (12). The purified proteins were analyzed on polyacrylamide gels as described (14), but with the addition of Triton X-100 at a concentration of 0.22%. The gels (1.5 mm thick) were preelectrophoresed at 25 mA for 2–3 hr at room temperature and electrophoresed at 250 V and 20 mA until the blue component of the methyl green tracking dye had reached the bottom of the gel (≈7 hr for a 20-cm gel). Gels were stained for 30 min with Coomassie blue and destained as described (14).

After destaining, the gels were photographed and either scanned with a gel densitometer (E-C), and then sliced for assay in experiments involving [^{3}H]acetate and ^{32}P or, alternatively, dried and then autoradiographed with x-ray film for the times desired in experiments involving ^{32}P.

[^{3}H]Acetate Labeling of the Cells. For a typical acetylation experiment, 10^7 cells were grown in monolayer in a T-150 flask. Cycloheximide was added for 30 min at a final concentration of 10 μg/ml followed by 2 mCi (1 Ci = 3.7×10^{10} becquerels) of [^{3}H]acetate (2 Ci/mmol) for 30 min at 37°C. The radioactive medium was then removed and replaced with fresh medium containing 50 mM sodium butyrate. Cells were incubated for 1 hr at 37°C before harvesting.

[^{32}P]Orthophosphate Labeling of the Cells. In a typical phosphorylation experiment, a T-150 flask containing 10^7 cells was used. The medium was gently removed and replaced with 10 ml of phosphate-free medium. Then, 1.5 mCi of [^{32}P]-orthophosphate was added and incubation was for 90 min at 37°C. The radioactive medium was then removed, and the cells were washed once in phosphate-free medium and then incubated for 2 hr in phosphate-free medium containing 50 mM sodium butyrate. In a control experiment, treatment with sodium butyrate was omitted.

Alkaline Phosphatase Assay. ^{32}P-Labeled samples (150 μg of protein per 9000 cpm of ^{32}P) were incubated with calf intestine alkaline phosphatase (2 μg; Boehringer Mannheim; 400 units/mg) for 90 min in 10 mM Tris·HCl, pH 8.0/1 mM $MgCl_2$/0.1 mM $ZnCl_2$/1 mM phenylmethylsulfonyl fluoride. The reaction was stopped by freezing the tubes.

Snake Venom Phosphodiesterase Assay. ^{32}P-Labeled samples (150 μg of protein/9000 cpm of ^{32}P) were incubated with 2 μg of snake venom phosphodiesterase (Boehringer Mannheim) for 3 hr at 37°C in 20 mM Tris·HCl, pH 8.0/1.5 mM $MgCl_2$/1 mM phenylmethylsulfonyl fluoride.

RESULTS

Protein Content of Chromatin Fractions Derived by Micrococcal Nuclease Treatment of Control and Butyrate-Treated HeLa Cell Nuclei. Nuclei derived from either control or butyrate-treated HeLa cells were separated into micrococcal nuclease-sensitive and -resistant chromatin fractions. In a typical experiment, among more than 30 performed, 10–20% of the input A_{260} and corresponding to <5% acid soluble, was recovered in fraction S_1, 20–40% was in fraction S_2 (>95% of this material is acid insoluble), and 40–70% was found in the pellet. S_2 was further fractionated to yield MN_1 and MN_2 (12, 13). In most preparations, in which 30% of the A_{260} was found in S_2, about half of it was recovered in MN_1 and the other half was in MN_2. These results were similar in both control and butyrate-treated cells.

The composition of acid-soluble proteins from each one of these fractions is illustrated in Fig. 1. The overall protein content of the corresponding fractions was similar for control and butyrate-treated cells. Fraction S_1 contained some H2A and H2B and a series of nonhistone proteins, among which the most prominent was ubiquitin (15). Traces of HMG-14 and -17 were also seen. On the other hand, MN_1 contained the four inner nucleosomal histones H2A, H2B, H3, and H4 together with a high content of the two smaller HMG proteins, HMG-14 and -17, and protein A24 (16). It is important to stress that most of the HMG-14 and -17 content of HeLa cell chromatin was found in fraction MN_1 in both control and butyrate-treated cells. Fraction MN_2 contained mainly the five histones plus A24 and traces of HMG-14 and -17. The pellet contained the five histones, smaller amounts of other unidentified non-histone proteins, and no HMG.

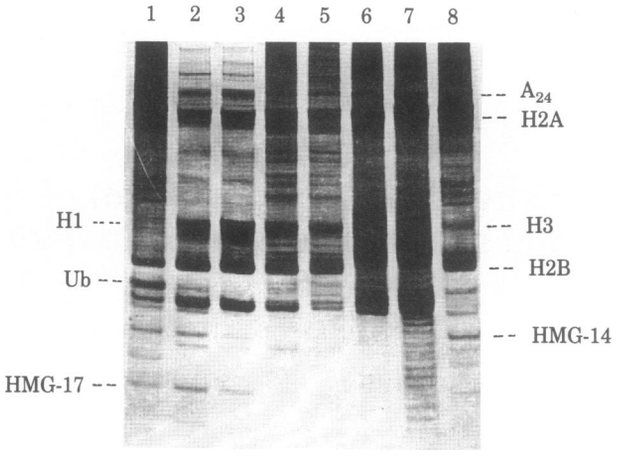

FIG. 1. Triton/acid/urea/polyacrylamide gel electrophoresis of acid-soluble proteins from chromatin fractions derived from butyrate-treated or control HeLa cells. Lanes: 1, S_1 from control cells; 2, MN_1 from control cells; 3, MN_2 from control cells; 4, pellet from control cells; 5, pellet from butyrate-treated cells; 6, MN_2 from butyrate-treated cells; 7, MN_1 from butyrate-treated cells; 8, S_1 from butyrate-treated cells. Ub, ubiquitin.

The major difference in protein composition of the various chromatin fractions between control and butyrate-treated cells was the presence of a series of more slowly migrating bands, corresponding to modified species of histones H2B, H3, and H4 and HMG-14 and -17, in the butyrate-treated samples (Fig. 1, lane 7). In the case of H2B, H3, and H4, these bands reflect multiacetylated species which have been shown to accumulate in butyrate-treated cells (2, 3) due to inhibition of histone deacetylase (4–6). However, to date, there is no evidence that HMG-14 and -17 can be modified by acetylation. Thus, it was surprising to find the series of bands that appeared in the HMG-14 and -17 regions of the gel in fraction MN_1 from butyrate-treated cells.

In view of this observation, I examined the possibility that the multiple bands observed might correspond to multiacetylated forms of HMG-14 and -17 which might have arisen by the same butyrate inhibition of the deacetylase enzyme.

Are HMG-14 and -17 Hyperacetylated After Butyrate Treatment of HeLa Cells? To approach this question, HeLa cells were treated with cycloheximide to terminate protein synthesis and eliminate the possibility of incorporation of [^{3}H]acetate into newly synthesized histone, either as amino acid residues or as a permanent NH_2-terminal modification (17). These cells were labeled with [^{3}H]acetate. Butyrate was then added to block histone deacetylation. Nuclei were prepared and acid-soluble proteins were extracted. Fig. 2 shows electrophoretic profiles of the [^{3}H]acetate-labeled nuclear proteins from butyrate-treated cells. Radioactive acetate was associated with histones H2B, H3, and H4 and with the large HMG protein HMG-1 (18). No labeled acetate was found in the region of HMG-14 and -17. Essentially the same results were obtained when the labeling was for 60 min instead of 30 min with cells exposed to

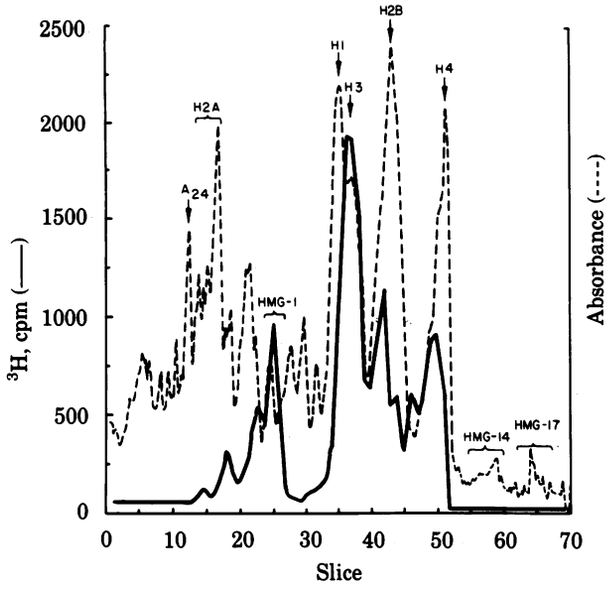

FIG. 2. Triton/polyacrylamide gel electrophoresis profile of proteins labeled with [^{3}H]acetate.

5 mM butyrate for 20 hr prior to labeling with acetate or chased with 20 mM butyrate for 2 hr after labeling.

Thus, under conditions in which the histones and large HMGs are readily acetylated, I failed to observe any incorporation of [^{3}H]acetate into the smallest HMG proteins, HMG-14 and -17, from HeLa cells. These data suggest that HMG-14 and -17 are not acetylated in HeLa cells or, if they are acetylated, their levels of acetylation are too low or the turnover rate is too rapid for acetylation to be detectable.

The original question remains as to the cause of the multiple banding pattern of HMG-14 and -17 derived from butyrate-treated cells. Having discarded acetylation as a cause, I examined the possibility that the HMG-14 and -17 might be modified by phosphorylation, which would also promote slower migration in acid/urea gels.

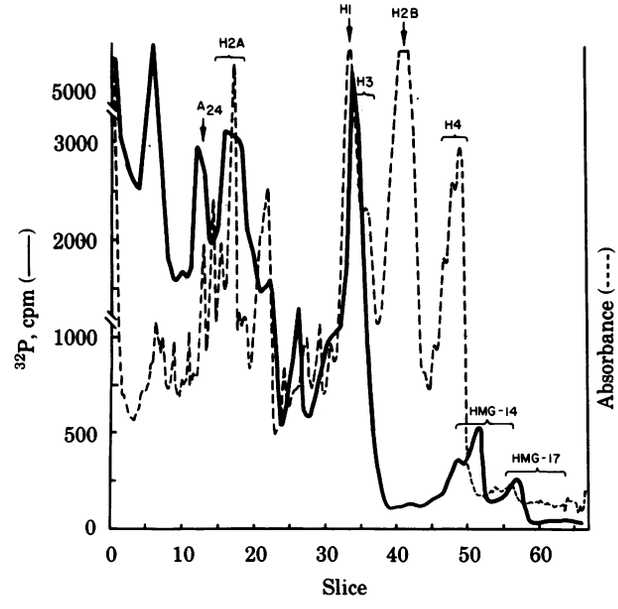

FIG. 3. Triton/polyacrylamide gel electrophoresis profile of proteins labeled with [^{32}P]orthophosphate.

Phosphorylation Experiments. Nuclei were prepared and acid-soluble proteins were extracted as described above. The electrophoretic profile of the ^{32}P-labeled proteins is illustrated in Fig. 3. Histones H2A, H3, and A24, but not H1, incorporated radioactive phosphate groups. These data agree well with those of D'Anna *et al.* (19) for Chinese hamster ovary cells, showing that butyrate selectively promotes the phosphorylation of H2A and, to a lesser extent of H3 and causes the dephosphorylation of H1.

Furthermore, ^{32}P label also was found associated with stained bands migrating slightly behind the parental forms of HMG-14 and -17, suggesting that these two proteins had become phosphorylated. That the ^{32}P label does not simply adhere to the proteins was shown by mixing purified proteins with an excess of [^{32}P]orthophosphate just prior to electrophoresis. In this case, no labeled material was found associated with any of the proteins. The covalent nature of the linkage of the [^{32}P]orthophosphate to the proteins was confirmed by testing its sensitivity to alkaline phosphatase (Fig. 4). The ^{32}P label associated with HMG-14 and -17 disappeared after treatment with alkaline phosphatase, thus confirming that the phosphate groups were covalently bound to protein.

These results show that, in HeLa cells, sodium butyrate enhances the phosphorylation of HMG-14 and -17. These proteins were phosphorylated to a lesser extent (one-fifth to one-third lower) when the sodium butyrate treatment was omitted.

[^{32}P]Orthophosphate can also be incorporated into ADP-ribose residues. Because such residues are known to be incorporated into the smallest HMG from trout testis, H6, analogous to mammalian HMG-14 and -17 (20), it was necessary to consider such a secondary modification of HMG-14 and -17 as the basis for ^{32}P labeling. To this end, I made use of the fact that the labeled phosphate in ADP-ribose groups is sensitive to digestion by snake venom phosphodiesterase (21). An aliquot of ^{32}P-labeled protein was incubated with this enzyme prior to electrophoresis and autoradiography. The results (Fig. 4, lane 7) show that snake venom phosphodiesterase failed to remove the labeled groups from HMG-14 and -17, thus arguing against poly(ADP-ribosylation). Nevertheless, it would appear that some of the ^{32}P label of HMG-17, but not of HMG-14, might have been removed, suggesting that a portion of the ^{32}P label in HMG-17 might correspond to poly(ADP-ribose) groups.

DISCUSSION

I have analyzed the composition of acid-soluble proteins from transcriptionally competent and inert chromatin fractions generated by controlled micrococcal nuclease action on nuclei from butyrate-treated or control HeLa cells. Most of the smaller HMG proteins, HMG-14 and -17, were localized in the salt-soluble mononucleosome fraction MN_1 (Fig. 1), whether control or butyrate-treated nuclei were analyzed. This observation is analogous to that reported for trout testis (12) in which it was shown that the trout HMG protein H6, homologous to mammalian HMG-14 and -17, is localized preferentially in fraction MN_1 prepared in the same manner. Furthermore, a series of bands with electrophoretic mobilities slower than those of the corresponding parental bands were displayed when HMG-14 and -17 in fraction MN_1 of butyrate-treated cells were analyzed on acid/urea/polyacrylamide gels.

In an attempt to determine the basis for the slowly migrating HMG bands, I entertained the possibility that butyrate might cause hyperacetylation of the HMG proteins in a manner analogous to histone hyperacetylation brought about by inhibition of deacetylase activity. Therefore, various experiments were performed to determine whether HMG-14 and -17 are susceptible to modification by acetylation and whether sodium buty-

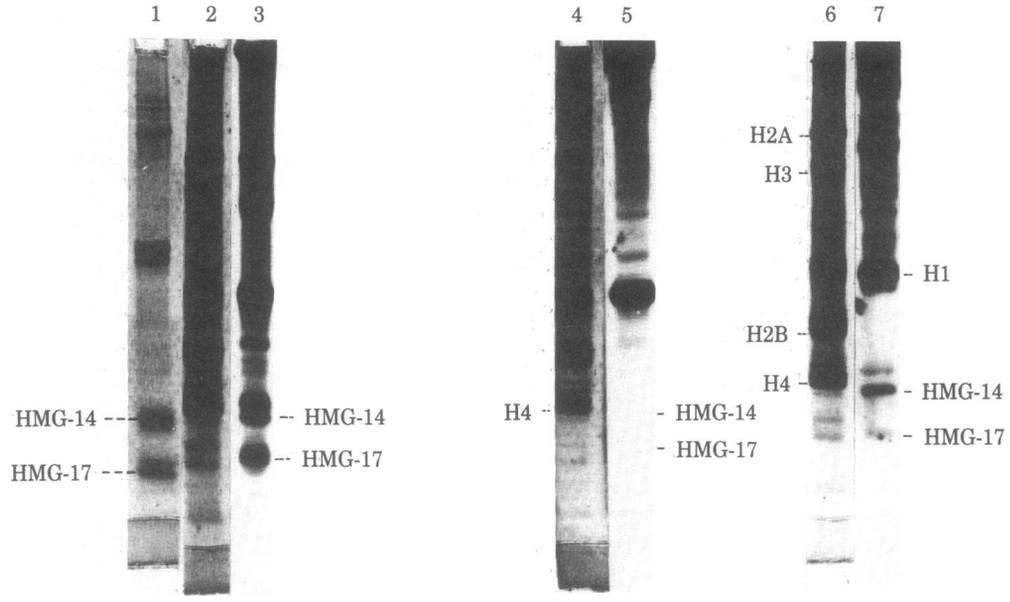

FIG. 4. Analysis of phosphorylated proteins. Lane 1, purified calf thymus HMGs used as markers. Lane 2, stained gel containing acid-soluble proteins labeled with ^{32}P. Lane 3, autoradiogram of the labeled material in lane 2. Lanes 4 and 5, alkaline phosphatase treatment of an aliquot of the sample in lane 2 (lane 4 shows the stained gel; lane 5 is the corresponding autoradiogram). Lanes 6 and 7, snake venom phosphodiesterase treatment of an aliquot of the sample in lane 2 (lane 6 is the stained gel; lane 7 is the corresponding autoradiogram).

rate enhances this effect. The results of all such experiments failed to provide evidence that HMG-14 and -17 from HeLa cells are acetylated. The levels of acetylation must be at least 10 times lower than those in the histones under the same experimental conditions. Alternatively, the turnover rate of the acetyl groups in HMGs may be so fast that they are undetectable.

In view of the above results, I examined phosphorylation of HMG-14 and -17. To this end, the incorporation of ^{32}P-labeled acid-soluble proteins from HeLa cells was analyzed. The results, illustrated in Figs. 3 and 4, permit several conclusions. First, HMG-14 and -17 are labeled when HeLa cells are exposed to [^{32}P]orthophosphate. The linkage is covalent as judged by its susceptibility to alkaline phosphatase. The multipeak distribution of stained HMG-14 and -17 together with the breadth of the labeled peaks suggests the possibility of more than one phosphate group per molecule of HMG. The modification does not appear to be ADP-ribosylation, as judged by the insensitivity of the ^{32}P-labeled groups to digestion by snake venom phosphodiesterase (21). Unequivocal proof that HMG-14 and -17 are phosphorylated will come from the identification of the modified residue. Nevertheless, the evidence presented in this manuscript strongly suggests that this is the case. Recent work by Saffer and Glazer (22) demonstrates that, in Ehrlich ascites cells and L1210 cells, HMG-14 and -17 readily incorporate [^{32}P]orthophosphate, thus providing support for my data.

Furthermore, most of the HMG-14 and -17 are localized in fraction MN_1 corresponding to those mononucleosomes most sensitive to micrococcal nuclease and recently found to be highly enriched in transcribed DNA sequences (9), implying that phosphorylation of the two small HMG proteins might play some role in the regulation of gene activity.

The magnitude of the phosphorylation effect is enhanced by sodium butyrate, suggesting various possible regulatory mechanisms. For instance, it is known that, in some human cell lines in culture, sodium butyrate increases the intracellular levels of cyclic AMP (1). Thus, it is conceivable that some of the effects of sodium butyrate in part may be mediated by cyclic AMP. There is ample evidence that cyclic AMP at low concentrations stimulates the phosphorylation of histones (22). This effect is due in some systems to an activation of specific histone phosphokinases (23, 24). Perhaps the kinase that phosphorylates histones may also utilize HMG-14 and -17 as substrates. Alternatively, high concentrations of sodium butyrate may inhibit the turnover of phosphate groups in these proteins by selective inhibition of the phosphatases.

Recently, Whitlock *et al.* (25) have reported that pretreatment of HeLa cells with sodium butyrate selectively increases the susceptibility of histone H3 to phosphorylation by a Ca^{2+}-dependent protein kinase, opening the possibility that a similar mechanism might operate in the butyrate-enhanced phosphorylation of HMG-14 and -17.

I thank Miss Patti Egan for her assistance in maintaining and labeling the HeLa cell cultures. I am grateful to Professor E. K. Wagner and his technicians, Beverly Gaylord and Lori Tribble, for providing me with the starter HeLa cell cultures, for their advice on how to maintain the cell line, and for their kind gifts of [^{32}P]orthophosphate. I also thank Dr. Graham Goodwin for providing me with purified calf thymus HMGs used as markers on the gels and Virginia Baker for typing this manuscript. This research was supported by grants from the Anna Fuller Fund and the National Institutes of Health.

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