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Selective Stimulation of Nonhistone Chromatin Protein Synthesis in Lymphoid Cells by Phytohemagglutinin†

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ABSTRACT: The chromatin proteins synthesized by phytohemagglutinin-stimulated lymphocytes have been investigated using a double isotope labeling approach. Lymphocyte chromatin was dissociated in 0.4 M guanidine-HCl, 6 M urea, 0.1% β-mercaptoethanol, and 0.1 M sodium phosphate buffer at pH 7.0, which solubilized >90% of the total chromatin proteins. The histones and nonhistones were then separated from one another by ion exchange chromatography and analyzed by polyacrylamide gel electrophoresis. The nonhistone chromatin proteins accounted for less than 15% of the total protein in lymphocyte chromatin but they contained >70% of the [^{3}H]leucine incorporated into the chromatin protein during a 2-hr pulse. When lymphocytes were stimulated by phytohemagglutinin, there was a prompt increase in the synthesis of all the nonhistone proteins, but not of histones. Among the nonhistones, several specific proteins were preferentially synthesized during the activation of the cell by phytohemagglutinin.

The nonhistone, or acidic, chromatin proteins have been implicated in control of gene expression in eukaryotic organisms. This view is based primarily on demonstrations that nonhistone chromatin proteins show tissue specificity (Elgin and Bonner, 1970; Levy *et al.*, 1972), are present in increased amounts in metabolically active tissues (Dingman and Sporn, 1964; Bonner *et al.*, 1968) and euchromatin (Frenster, 1965) and can increase *in vitro* template activity of various preparations of chromatin for RNA polymerases (Gilmour and Paul, 1969; Wang, 1970). Specifically, nonhistone chromatin proteins have been suggested as a controlling element in the gene activation which occurs prior to mitosis in animal cells. For recent reviews see Stellwagen and Cole (1969a) and Stein and Baserga (1970).

Recently, we have developed a general method for the fractionation of chromatin (Levy *et al.*, 1972). Utilizing this method, nucleic acids, histones, and nonhistone proteins are separated from one another and quantitatively recovered from a single chromatin sample. We have now applied this methodology to extend our previous studies on the proteins of stimulated lymphocytes (Levy and Rosenberg, 1972a,b; Rosenberg and Levy, 1972). We have found that an increase in the rate of synthesis of nonhistone chromatin proteins is one of the earliest processes during the activation of the lymphoid cell by phytohemagglutinin. Moreover, among these proteins, certain specific nonhistones are preferentially stimulated.

Experimental Section

Lymphoid Cell Culture. The mesenteric lymph nodes of strain 2 guinea pigs (Animal Production Unit, National Institutes of Health) were removed, dissected free of fat and connective tissue, and gently teased apart with fine tipped forceps into tissue culture medium (Eagle's minimal essential medium lacking leucine but supplemented with penicillin (100 u/ml), streptomycin (100 μg/ml), glutamine (0.59 mg/ml), and nonessential amino acids). The larger particles were allowed to settle for 2 min and the lymphocytes in the supernatant were collected by centrifugation at 300*g* at room temperature, washed once, and diluted to a concentration of 10^7 cells/ml in leucine-free medium. Cell suspensions were cultured at 37° in a humidified atmosphere of 10% CO_2 in air. For considerations of geometry and gas equilibration, these incubations were conducted in 1-ml aliquots in round bottom 16 × 125 mm tubes.

PHA[1] Stimulation and Isotope Labeling. Phytohemagglutinin was dissolved at 100 μg/ml in a sterile solution of 0.14 M NaCl buffered at pH 7.2 with 0.007 M sodium phosphate and 0.1 ml was added to half the cultures. The other half of the cultures received the same volume of buffered saline alone. At various times thereafter, PHA and control cultures were

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[1] Abbreviation used is: PHA, phytohemagglutinin.

pulse labeled for 2 hr with different isotopes of radioactive L-leucine. For example, 100 μCi of [^{3}H]leucine (2.0 Ci/mmol) was added to the control cultures and 5 μCi of [^{14}C]leucine (312 mCi/mmol) was added to the PHA-stimulated cultures.

After the labeling period, the PHA and control cultures were combined and the cells were washed three times with Eagles medium containing leucine at 4° to remove unincorporated radioactive leucine.

Chromatin Preparation and Fractionation. Nuclei were isolated by a modification of the method of Hymer and Kuff (1964). The cell pellet was homogenized at 4° in buffer A (0.25 M sucrose–3 mM $CaCl_2$–0.01 M Tris-HCl, pH 8.0) containing 0.5% Triton X-100 and recentrifuged and the final nuclear pellet was washed once in buffer A.

Chromatin was prepared from the isolated nuclei by extraction at 4° with solutions of decreasing ionic strength from 0.05 to 0.001 M Tris-HCl, pH 8.0, essentially as described by Huang and Huang (1969). Each extraction was performed by gentle homogenization in a loose fitting ground glass homogenizer followed by centrifugation af 3000*g* for 15 min except for the final two centrifugations which were at 10,000*g*. The final chromatin pellet was homogenized in H_2O. Absorbance at 260 nm was used to estimate the DNA concentration, assuming the absorbance of 1 mg/ml of DNA to be 21.0 in 0.2% sodium dodecyl sulfate. In a typical experiment, 8×10^6 cells yielded chromatin containing 3.75 mg of DNA. The chromatin was then fractionated as previously described (Levy *et al.*, 1972).

Chromatin at a DNA concentration of 0.25 mg/ml was dissociated in 6 M urea, 0.4 M guanidine hydrochloride, 0.1% β-mercaptoethanol, and 0.1 M sodium phosphate buffer, pH 7.0, and the DNA was sedimented by centrifugation at 100,000*g* for 18 hr at 4°. Over 90% of the radioactivity of the chromatin was recovered in the supernatant. The guanidine hydrochloride concentration of the supernatant was adjusted to 0.35 M and it was applied to a column of the cation exchange resin Bio-Rex 70 (similar to IRC-50) equilibrated with 6 M urea–0.35 M guanidine hydrochloride–0.1 M phosphate, pH 7.0. The histones were adsorbed to the column, allowing their separation from nonhistones which pass through unretarded. The histones were then eluted by raising the guanidine concentration of the buffer to 3.0 M. Recovery of radioactivity from this column was greater than 85%.

Polyacrylamide Gel Electrophoresis. The method of Panyim and Chalkley (1969) was used for electrophoresis in urea at pH 2.7 and that of Weber and Osborn (1969) was employed for electrophoresis in the presence of sodium dodecyl sulfate, except that the molarity of the running and gel buffers was lowered to 0.03 M phosphate. Gels were stained with 1% Amido Black in 7% (v/v) acetic acid–40% (v/v) ethanol, destained in the same concentrations of acetic acid and ethanol in the presence of Dowex 1-X2 resin, and scanned at 550 nm in a Beckman Acta III spectrophotometer equipped with a densitometric scanner attachment. For radioactive counting, unstained gels were frozen at −70° and then sliced into 1-mm transverse sections with a device containing parallel steel blades imbedded in a plastic casing. Slices were transferred to scintillation vials, 0.2 ml of 0.5 N NaOH was added, and the capped vials were placed at 80° for 1 hr and then at room temperature for 12 hr. One milliliter of NCS solubilizer and 10 ml of Liquofluor–toluene (1:24) were then added and the samples were counted for ^{3}H and ^{14}C in a Packard Tri-Carb instrument. Recovery of radioactivity from gel slices by this method was greater than 80% and the same for ^{3}H and ^{14}C.

Data on paper tape were corrected for background and carry-over using an IBM 1620 computer interfaced to a Calcomp plotter which displayed ^{14}C and ^{3}H and their ratios directly.

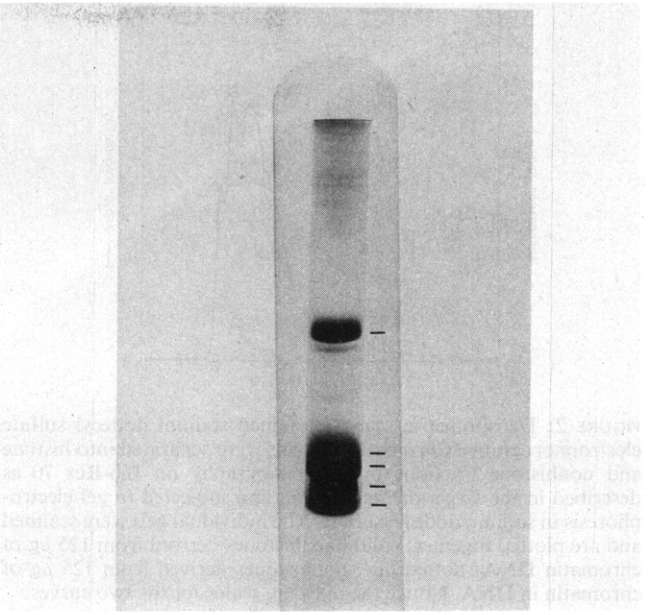

FIGURE 1: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of total chromatin proteins. Chromatin proteins were dissociated from DNA as described in the Experimental Section, dialyzed against H_2O, lyophilized, and prepared for electrophoresis by dissolution in 1% sodium dodecyl sulfate–1% β-mercaptoethanol–0.01 M PO_4, pH 7.2, and dialysis against 0.1% sodium dodecyl sulfate–0.1% β-mercaptoethanol–0.001 M PO_4, pH 7.2. The sample was derived from 150 μg of chromatin DNA. Direction of migration is from top (−) to bottom (+). Histone fractions are indicated from top to bottom: F_1, F_3, F_{2b}, F_{2a2}, F_{2a1}.

Sources of Chemicals. Sucrose, urea, and guanidine hydrochloride (Ultra Pure grade) were obtained from Schwarz-Mann Corp., specially purified sodium dodecyl sulfate was purchased from B.D.H. Corp., and Bio-Rex 70 (200–400 mesh sodium form, 10.2 mequiv/g) was purchased from Bio-Rad Laboratories. Triton X-100 and Liquifluor were obtained from New England Nuclear Corp. NCS solubilizer was purchased from Amersham/Searle Co. Toluene (scintillation grade) was purchased from Fisher Scientific. Nonessential amino acid mixture was purchased from Microbiological Associates, Bethesda Md., and purified phytohemagglutinin was purchased from Burroughs Wellcome Co., Research Triangle Park, N. C. Eagles minimal essential medium with and without leucine was prepared by the Media Unit, National Institutes of Health.

Results

Lymphocyte Chromatin Proteins. The sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern of the dissociated chromatin proteins is shown in Figure 1. The histones constitute the major protein bands but a group of higher molecular weight bands of nonhistone proteins can be seen.

Histones and nonhistones were separated from each other by ion exchange chromatography on Bio-Rex 70 and analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Figure 2) and in urea at pH 2.7 (Figure 3). From the area under these curves, we estimate that approximately 85% of the stainable protein of this chromatin is histone, a figure which agreed well with Lowry protein determinations of the two Bio-Rex 70 fractions. The gels also indicate the degree of

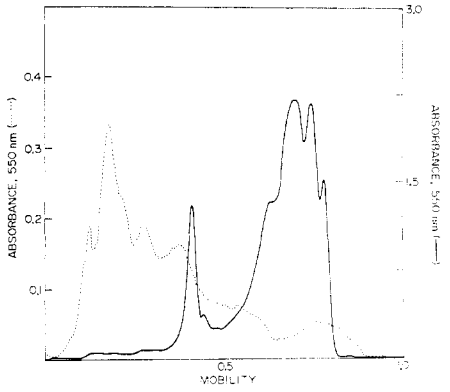

FIGURE 2: Densitometric scans of stained sodium dodecyl sulfate electrophorograms. Chromatin proteins were separated into histone and nonhistone fractions by chromatography on Bio-Rex 70 as described in the Experimental Section and subjected to gel electrophoresis in sodium dodecyl sulfate. The individual gels were scanned and are plotted together: solid line, histones derived from 125 μg of chromatin DNA; dotted line, nonhistones derived from 125 μg of chromatin in DNA. Notice the differing scales for the two curves.

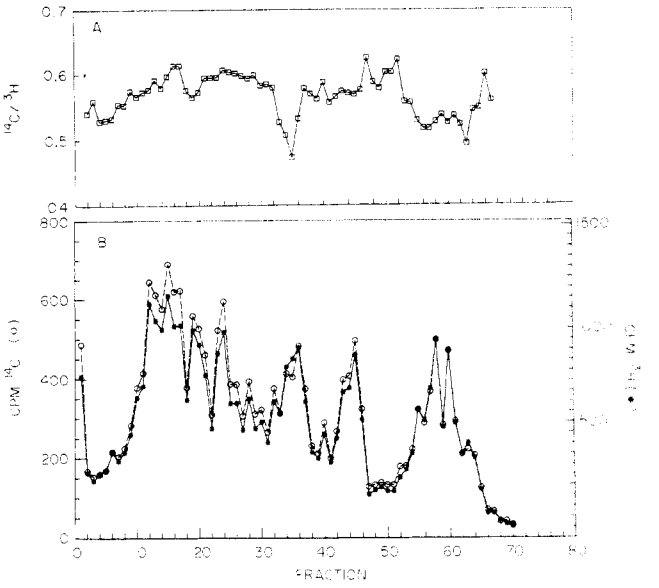

FIGURE 4: Sodium dodecyl sulfate gel electrophoresis of chromatin proteins from PHA-stimulated cells. Cells were incubated in the presence or absence of PHA, 10 μg/ml. One hour after the start of incubation the cultures were pulse labeled for 2 hr with either [^{14}C]leucine (PHA group) or [^{3}H]leucine (control group). The cells were pooled and chromatin was prepared, dissociated and, subjected to sodium dodecyl sulfate gel electrophoresis: (A) ^{14}C/^{3}H ratio of each fraction; (B) distribution of ^{14}C and ^{3}H. Histone bands were found in positions corresponding to fractions 33–36 and 53–65.

separation between the low molecular weight, basic histones and the higher molecular weight, acidic nonhistones. We have previously reported similar results for the fractionation of chromatin from rabbit spleen, kidney, and liver (Levy *et al.*, 1972).

PHA Stimulation. The lymphoid cells were cultured in the presence or absence of PHA, and pulse labeled for 2 hr with either [^{14}C]leucine (PHA group) or [^{3}H]leucine (control group). Separation of the combined chromatin into histones and nonhistones by ion exchange chromatography revealed that the nonhistone fraction, less than 15% of the total chromatin protein, contained 70% of the combined radioactivity. The total chromatin proteins were analyzed by sodium dodecyl sulfate gel electrophoresis as shown in Figure 4. Panel B contains the actual ^{3}H and ^{14}C counts and panel A shows the ^{14}C/^{3}H ratio for each fraction. In this experiment, the cells were labeled from 1 to 3 hr after PHA stimulation. At this early time, no PHA stimulation of leucine incorporation into total cellular Cl_3CCOOH precipitable material was detected. The ^{14}C/^{3}H ratio of the original labels as used was 0.47. It can be seen that the ratio of ^{14}C/^{3}H in the histone fractions (33–36, 53–65) did not differ significantly from 0.47, whereas the nonhistones as a group showed definite increases in their rates of synthesis as a result of PHA stimulation. The nonhistone proteins contained in fractions 16–17, 20–30, and 46–51 demonstrated a 20% increase in their synthesis relative to that in unstimulated cells. A similar distribution of radioactivity and an identical pattern of ^{3}H/^{14}C ratio was seen when the experiment was performed with the labels reversed, eliminating isotope effects or quenching artifacts as causes for the increases in radioactivity.

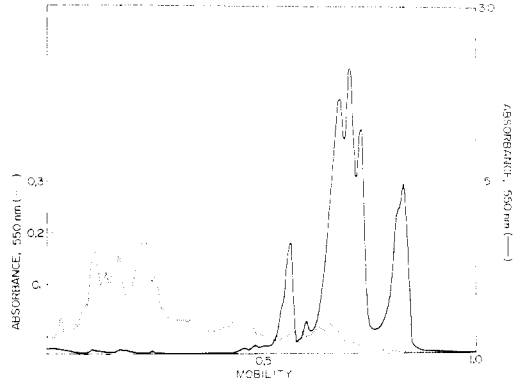

FIGURE 3: Densitometric scans of stained, pH 2.7, urea electrophorograms. Samples of Bio-Rex purified histones and nonhistones were subjected to polyacrylamide gel electrophoresis in urea at pH 2.7: solid line, histones derived from 125 μg of chromatin DNA; dotted line, nonhistones derived from 125 μg of chromatin DNA. Notice the differing scales for the two curves.

These preferential effects on the synthesis of nonhistone chromatin proteins were greatly increased 8 hr after PHA stimulation, as illustrated in Figure 5. Again, the PHA stimulated cells received [^{14}C]leucine and the nonstimulated cells received [^{3}H]leucine. The upper curve of ^{14}C/^{3}H in Figure 5A represents the data from this experiment and is derived from the gel shown in Figure 5B. The lower curve in Figure 5A is from a control experiment in which neither the cells pulsed with [^{14}C]leucine nor those labeled with [^{3}H]leucine were stimulated by PHA. In this experiment PHA stimulated the incorporation of leucine into the Cl_3CCOOH-soluble intracellular pool by a factor of 2.01, and into the total cellular Cl_3CCOOH precipitable proteins by a factor of 3.1. However, a selective stimulation of the synthesis of nonhistone nuclear proteins was observed. Proteins in fractions 14, 26, 40, and 52–54 showed approximately a sixfold stimulation in their rates of synthesis over the control. The positions of F_1 histone, fraction 39, and the other histones (fractions 59–70) were marked by relatively low ^{14}C/^{3}H ratios. This pattern of radioactivity and positions of ^{14}C/^{3}H ratio peaks shown in Figure 5 were remarkably consistent in repeated experiments.

These chromatin proteins were fractionated into nonhistones and histones and electrophoresed in parallel with the unfractionated chromatin proteins. The histones (Figure 6)

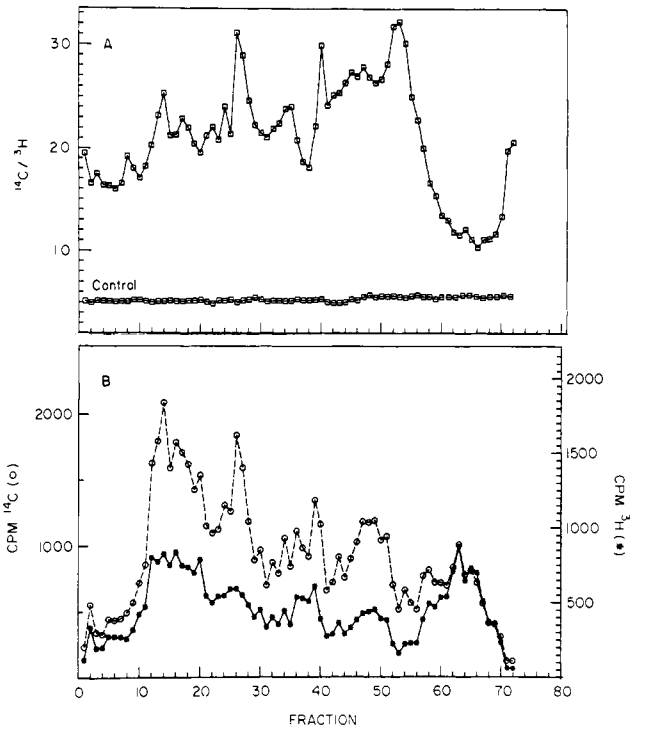

FIGURE 5: Sodium dodecyl sulfate gel electrophoresis similar to Figure 4 except that cultures were labeled for a 2-hr period beginning 8 hr after the addition of PHA: (A) upper curve derived from data in B; lower curve from a control experiment in which neither the cultures labeled with [^{14}C]leucine nor those labeled with [^{3}H]-leucine were stimulated with PHA; (B) distribution of ^{14}C and ^{3}H. The positions of histone bands corresponded to fractions 36–39 and 59–70.

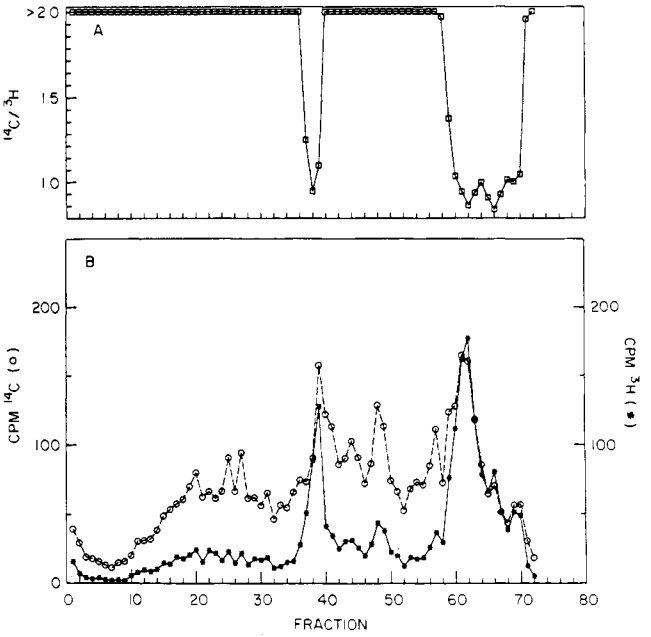

FIGURE 6: Sodium dodecyl sulfate gel electrophoresis of purified histones. Histones were purified from chromatin proteins shown in Figure 5 by chromatography on Bio-Rex 70 and subjected to sodium dodecyl sulfate gel electrophoresis: (A) ^{14}C/^{3}H ratios, plotted such that nonhistones are off scale; (B) distribution of ^{14}C and ^{3}H.

were contaminated slightly with nonhistone proteins. For this reason, as well as the unsteady ratios derived from low numbers of counts in the nonhistone areas, Figure 6A is plotted such that the nonhistone ratios fall above the scale and serves primarily to emphasize the positions of the histones and their low ^{14}C/^{3}H ratios. Some reciprocal contamination of histones was observed in the nonhistone sample and was reflected in the low ratios in fractions 60–70 in Figure 7A. More importantly, the ^{14}C/^{3}H ratios of the nonhistone fraction contained all of the peaks seen in the total chromatin (Figure 5A). An additional peak, fraction 33–36, was accentuated by the removal of the closely positioned F_1 histone.

Discussion

The PHA-stimulated lymphocyte is a good model system for studying the transition of a mammalian cell from a quiescent to a metabolically active, dividing state. DNA synthesis and mitosis occur in these cells 24–48 hr after PHA stimulation but a number of earlier biochemical events have been reported. We have previously described a number of changes in cellular proteins which occur within the first several hours of this activation process (Rosenberg and Levy, 1972; Levy and Rosenberg, 1972a,b). Several studies have demonstrated structural and functional changes in nuclear chromatin early after PHA stimulation. Acetylation of arginine-rich histones (Pogo *et al.*, 1966), increased exchange of phosphate residues in nuclear proteins (Kleinsmith *et al.*, 1966), exposure of Acridine Orange binding sites in the nucleus (Killander and Rigler, 1965), and an increased template activity of nuclei for bacterial RNA polymerase (Hirschhorn *et al.*, 1969) have all been demonstrated to occur as early events.

The present studies indicate that another early event in the activation of lymphoid cells by PHA is the stimulation of nonhistone chromatin protein synthesis. This effect was observed as early as 1–3 hr after the addition of PHA to the cultures (Figure 4). In addition, certain specific nonhistone proteins were preferentially produced during the process of cellular

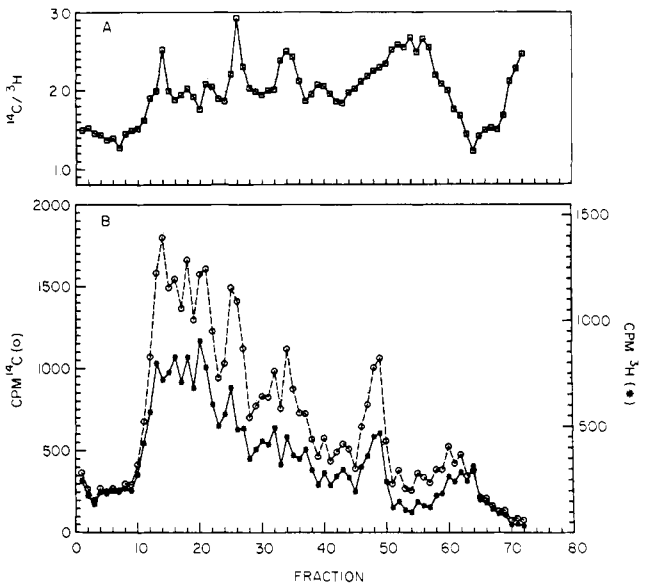

FIGURE 7: Sodium dodecyl sulfate gel electrophoresis of purified nonhistones. The nonhistone proteins were purified from the chromatin proteins shown in Figure 5 by chromatography on Bio-Rex 70 and subjected to sodium dodecyl sulfate gel electrophoresis: (A) ^{14}C/^{3}H ratios for the individual fractions; (B) distribution of ^{14}C and ^{3}H.

activation. This was more apparent by 8 hr after the addition of PHA (Figures 5 and 7).

Several other activated mammalian systems have been investigated and were found to display increases in nonhistone chromatin protein synthesis. Teng and Hamilton (1969) reported an increased *in vivo* uptake of amino acids into an acid-insoluble nucleoprotein fraction of the rat uterus after estrogen stimulation. Stellwagen and Cole (1969b) showed a similar effect on the rat mammary gland during the lactating state. Rovera and Baserga (1971) demonstrated a stimulation of amino acid incorporation into certain salt-extractable or acid-insoluble fractions of the nuclei of nutritionally stimulated WI-38 fibroblast cells. Stein and Baserga (1970) used the same nuclear extraction scheme for mouse salivary gland nuclei and reported the stimulation of specific nonhistone chromatin proteins in that tissue by isoproterenol. Finally, Shelton and Allfrey (1970) using a method involving phenol extraction found specific stimulation of a nonhistone nuclear protein of molecular weight 41,000 in cortisol-stimulated, rat liver cells.

Metabolic activation in preparation for mitosis of a mammalian cell must require the controlled expression of a large number of genes and we would expect to find great complexity in the molecular mechanisms which mediate this control. For this reason, our initial attempts to describe the role of acidic chromatin proteins in the regulation of gene activation of such systems were not focused on a small subfraction of these proteins. The approach used in the present study was based on a method of chromatin fractionation which separates the histones from the nonhistones but excludes none of the chromatin proteins from analysis. Furthermore, with this methodology, all of the other complementary nonprotein components of the same chromatin samples are available for study. Using a combination of the double label design described here with preparative scale fractionation methods, it should be possible to isolate and characterize particular nonhistone chromatin proteins from activated mammalian systems and, at the very least, to determine the degree to which they reflect the specificity of genetic expression.

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