Developmentally Regulated Nonhistone Proteins: Evidence for Deoxyribonucleic Acid Binding Role and Localization near Deoxyribonuclease I Sensitive Domains of Precartilage Cell Chromatin†

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ABSTRACT: Differentiation of cartilage from precartilage mesenchyme in the chick embryo is accompanied by the loss of two abundant nonhistone proteins (M_r 35 500 and 125 000) termed PCP 35.5 and PCP 125. Here we examine the distribution of these and other developmentally regulated nonhistones in nuclease-sensitive regions of precartilage and cartilage chromatin. In particular, we show that PCP 35.5 is a tight DNA-binding protein that is localized near deoxyribonuclease I (DNase I) sensitive regions of precartilage chromatin. Localization of nonhistones was demonstrated by excising domains of precartilage chromatin with DNase II which are simultaneously highly enriched in PCP 35.5, in PCP 125, and in DNase I sensitive DNA sequences. These domains comprise at least 25% of the cell's DNase I sensitive sequences, as well as small DNase I resistant regions with which the two nonhistones are associated. These findings suggest that PCP 35.5 (and possibly PCP 125) may play a developmentally regulated role nearby DNase I sensitive domains of the cartilage progenitor cell chromatin.

The regulation of cell differentiation during metazoan development probably involves factors or processes of a general nature that facilitate template activity in designated regions of chromatin DNA,[1] as well as cell type specific factors that determine which particular sequences in a given cell become activated. The first class may include the high mobility group nonhistone proteins (HMG's) 14 and 17, which impose a deoxyribonuclease I (DNase I) sensitive conformation on the nucleosomes of template-competent genes in a number of cell types (Weisbrod & Weintraub, 1979; Weisbrod et al., 1980) and demethylation of methylcytosines in the DNA of differentiating cells (Mandel & Chambon, 1979; Razin & Riggs, 1980). Less is known about the second class of regulatory factors, although those nonhistone chromatin proteins that are differentially distributed across cell types are obvious candidates for this role.

We have attempted to identify proteins that may be involved in cellular reprogramming during development by examining the chromatins of two lineage-adjacent cell types, the cartilage progenitor (or precartilage) cell (Newman, 1977; Newman et al., 1981) and the definitive cartilage cell of the embryonic chick.

A comparative study of these two chromatin types led to the finding that an abundant ($\sim 10^6$ copies per nucleus) nonhistone protein (M_r 125 000) is lost during cartilage differentiation in vitro, against a relatively unchanging background of other nonhistones (Newman et al., 1976). Further analysis has confirmed that this component, which we term PCP (precartilage chromatin protein) 125, is lost during chondrogenesis in vitro and that it is present in an altered form (M_r 120 000) in the precartilage cells of homozygotes of the chicken mutant *talpid*^{2}, which exhibit an aberrant spatial pattern of cartilage differentiation (Perle & Newman, 1980). Another abundant nonhistone of normal precartilage cells [(M_r 35 500) designated PCP 35.5] is also lost during chondrogenesis and is reduced in amount in *talpid*^{2} precartilage cells (Perle & Newman, 1980). More recently we have found that PCP 125 is a basic protein and that PCP 35.5 comprises several components with different p*I*'s, one of which is a target for cyclic AMP dependent phosphorylation in isolated precartilage nuclei (S. A. Newman and C. M. Leonard, unpublished results).

These studies have established that precartilage and cartilage cells, which synthesize very different arrays of cell type specific molecules (Linder et al., 1975; von der Mark & von der Mark, 1977; Vertel & Dorfman, 1978), differ in a small number of abundant chromatin proteins, at least two of which, PCP 35.5 and PCP 125, exhibit properties that are consistent with regulatory roles. In the present study we have made use of the chromatin structure recognition properties of several nucleases to determine the localization of PCP 35.5, PCP 125, and other nonhistones relative to the DNase I sensitive regions of precartilage and cartilage chromatin. We have found that PCP 35.5 and PCP 125 are not released from precartilage chromatin by DNase I but that major amounts of them are released from precartilage chromatin by DNase II as part of a chromatin subfraction, 90% of whose DNA is from the DNase I sensitive regions. Furthermore, PCP 35.5 is shown to be a tight DNA-binding protein of precartilage chromatin. It can therefore be presumed to coisolate with its native binding sequences in the fractionation protocols. These results imply that PCP 35.5 and possibly PCP 125 are part of a macromolecular structure contiguous with DNase I sensitive chromatin DNA in the precartilage cell and suggest that they may play a developmentally regulated role near these DNase I sensitive domains.

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[1] Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; DNase, deoxyribonuclease; RNase, ribonuclease; PCP, precartilage chromatin protein; NPB, nucleic preparation buffer; RSB, reticulocyte standard buffer; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; PCA, perchloric acid.

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Materials and Methods

Isolation of Nuclei. Precartilage mesenchyme was prepared from distal wing tips of 5-day-old white Leghorn chick embryos, cut at a distance of 0.3 mm and parallel to a tangent to the wing apex, as previously described (Newman et al., 1976; Perle & Newman, 1980). Embryonic cartilage was prepared from decapitated 7-day-old embryos by exhaustive washing in distilled water, following the procedure of Linsenmayer (1974).

Nuclei were prepared at 4 °C from both tissues by homogenization in nuclei preparation buffer (NPB: 0.01 M NaCl, 3 mM $CaCl_2$, 0.25 M sucrose, 0.01 M Tris, pH 7.4) containing 1 mM phenylmethanesulfonyl fluoride (PMSF) (added in 2-propanol), 5 mM sodium butyrate, and 0.5% Nonidet P-40 (Sigma) in a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was filtered through 20-μm Nytex monofilament nylon mesh and centrifuged at 6000 rpm in a Sorvall HB-4 rotor for 5 min. The pellet was washed in NPB containing PMSF and sodium butyrate and either used immediately or stored in the same buffer made up in 50% glycerol at −70 °C or in liquid nitrogen. Ten 5-day-old wing tips or cartilage rudiments from seven 7-day-old embryos yield about 1×10^6 nuclei.

Assay for Tight DNA-Binding Proteins. Proteins binding tightly to DNA in precartilage chromatin were assayed by a modification of the procedure of Pederson & Bhorjee (1975). Precartilage nuclei were prepared as described above, suspended in 1 mL of RSB (0.01 M Tris, pH 7.4, 0.01 M NaCl, 3 mM $MgCl_2$), and sonicated with two successive 15-s pulses at setting 3 with a Branson 200 sonifier. Two volumes of RSB containing 3.75 M NaCl and 4.75 M urea was added to the sonicate, which was then centrifuged at 36 000 rpm for 46 h in the Beckman 50 Ti rotor. Using precartilage cells radiolabeled in vivo with [^{3}H]thymidine, we have determined that this procedure pellets more than 98% of the DNA. The resulting pellet was dissolved directly in the $NaDodSO_4$ gel sample buffer containing 5 M urea (Perle & Newman, 1980). The supernatant was made 20% in trichloracetic acid, incubated for 15 min on ice, and centrifuged at 15 000 rpm for 10 min. The pelleted proteins were washed in 95% ethanol and diethyl ether, dried, and dissolved in sample buffer.

Enzyme Treatments of Nuclei and Chromatin. For DNase I digestions, 1×10^7 nuclei were suspended in 60 μL of RSB and 1 μL of 0.1 M PMSF. Two microliters of a 0.1 mg/mL solution of DNase I (Sigma; electrophoretically purified, 2000 units/mg) in water was added to the nuclear suspension, which was incubated at 37 °C. Aliquots were removed at successive time points, made 20 mM EDTA and 3.3 M urea, and placed on ice. Centrifugation at 11 000 rpm in the Sorvall HB-4 rotor yielded a pellet (P) and a supernatant (S). Where DNA solubilization was monitored, the S fraction was made 7% in perchloric acid (PCA) and the precipitable radioactivity was added to that of the P fraction. For DNase II digestions, 1×10^7 nuclei were suspended in 56 μL of 0.025 M sodium acetate, 1 mM $CaCl_2$, pH 6.6, and 1 μL of 0.1 M PMSF. Three microliters of a 1 mg/mL solution of DNase II (Worthington; chromatographically purified, 18 500 units/mg) was added to the chromatin suspension, which was incubated at 37 °C. Aliquots were removed at successive time points, brought to pH 9.0 with Tris, and centrifuged at 11 000 rpm for 15 min. The pellet generated at this step is the P1 fraction. The supernatant was made 5 mM in $MgCl_2$ and placed on ice for 20 min. Centrifugation at 11 000 rpm for 15 min yielded a P2 (Mg^{2+} insoluble fraction) and S (Mg^{2+} soluble fraction) fraction (Gottesfeld et al., 1974, 1975). Where radioactive chromatin was fractionated by DNase I or DNase II, each pellet was boiled for 15 min in 260 μL of 7% perchloric acid. Each supernatant was brought to 260 μL of 7% PCA and also boiled for 15 min. Samples were mixed with 4 mL of Aquasol II (New England Nuclear) left overnight in the dark, and their radioactivity was determined in a liquid scintillation counter. Where radioactive P1 or P2 fractions were redigested with DNase I, each fraction was brought to the DNA equivalent of 1×10^7 nuclei by addition of nonradioactive unfractionated nuclei prior to DNase I digestion.

For RNase digestions, 5×10^6 nuclei were suspended in 100 μL of 0.025 M sodium acetate, 1 mM $CaCl_2$, and 1 mM PMSF and treated with a mixture of RNase A (Worthington; 100 units/mL) and RNase T_1 (Worthington; 100 units/mL) at 37 °C for 20 min. Incubation mixtures were made 20 mM EDTA and 3.3 M urea, placed on ice, and centrifuged for 15 min at 11 000 rpm. Resulting pellets and supernatants were prepared for gel electrophoresis as described below.

In Ovo Labeling of Nuclei. Fertile eggs were windowed at 2 days of incubation by candling, cutting an opening in the shell over the embryo, removing the shell membrane, and covering the opening with Parafilm. Eggs were incubated until 24 h before harvesting precartilage or cartilage tissue. At that point embryos were injected with 10–25 μCi of [6-^{3}H]thymidine (New England Nuclear, NET-355) delivered beneath the embryonic membranes in 0.3 mL of sterile balanced salt solution. The Parafilm was then resealed, and the eggs were allowed to incubate for another 24 h. Nuclei were prepared from each tissue as described above and mixed with the appropriate number of nonradioactive nuclei to make up a standard incubation mixture.

Labeling of Nuclei by Nick Translation. The method followed was essentially identical with that of Gazit et al. (1980). Precartilage nuclei (1×10^7) were suspended in 200 μL of nick translation buffer (50 mM Tris, pH 7.9, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 50 μg/mL bovine serum albumin) containing 40 ng of DNase I and incubated at 37 °C for 5 min. The nuclear suspension was then placed on ice and transferred to another tube containing the following dry nucleotides: 0.8 nmol of dATP, 0.8 nmol of dCTP, 0.8 nmol of dGTP, 0.6 nmol of dTTP, and 10 μCi of [^{3}H]dTTP (Amersham TRK576; 60–100 Ci/mmol). To this mixture was added 4 μL of *Escherichia coli* DNA polymerase I (86 units/mL), prepared by Dr. Susan Wallace according to the procedure of Jovin et al. (1969) and assayed with nicked single-strand DNA as described. This procedure yields an enzyme free of exonuclease III/endonuclease VI activity. Incubation was for varying times at 15 °C, after which nuclei were washed 3 times in nick translation buffer. After the experiment recorded in Figure 7A was performed, the standard incubation with DNA polymerase I was taken to be 10 min.

Gel Electrophoresis of Proteins. Nuclear pellets or pellets from nuclease fractionations were dissolved in a sample buffer consisting of 0.08 M Tris, pH 6.8, 0.1 M dithiothreitol, 2% sodium dodecyl sulfate ($NaDodSO_4$), 5 M urea, 10% glycerol, and 0.12% bromphenol blue by boiling for 2 min. Supernatants from nuclear fractionations were precipitated with 20% trichloroacetic acid. Precipitates were washed 2 times with 95% ethanol and 2 times with anhydrous diethyl ether, air-dried, and boiled in the same sample buffer. Electrophoresis was performed on $NaDodSO_4$–12.5% polyacrylamide slab gels by using the gel system of Laemmli (1970) with the acrylamide–methylenebis(acrylamide) ratio modified according to Blattler et al. (1972).

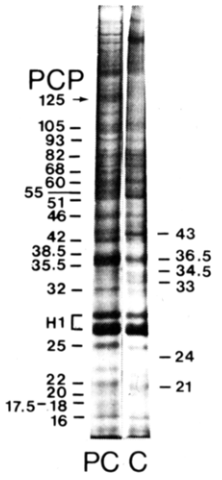

FIGURE 1: Comparison of precartilage and cartilage chromatin proteins by gel electrophoresis. Electrophoresis was on $NaDodSO_4$–12.5% polyacrylamide gels. Numbers represent $M_r/10^3$. PC, precartilage; C, cartilage.

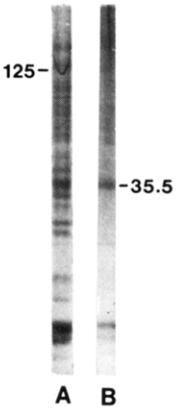

FIGURE 2: Proteins binding tightly to DNA in precartilage chromatin. Chromatin was dissolved in 2.5 M NaCl and 3.2 M urea, and the DNA and tight binding proteins were pelleted, as described under Materials and Methods. Electrophoresis was on $NaDodSO_4$–12.5% polyacrylamide gels. (A) Proteins in supernatant; (B) proteins tightly bound to DNA.

Molecular weights were determined by plotting distance from gel origin of stained bands vs. logarithm of molecular weight relative to the following standards of known subunit molecular weight: *Bacillus subtilis* RNA polymerase (a gift from Nathaniel Heintz), actin, tubulin, and bovine serum albumin. Molecular weights of chromatin proteins were determined on at least three gels for each experiment reported.

Results

Comparison of Nonhistone Proteins of Precartilage and Cartilage Nuclei. Figure 1 shows the patterns of nuclear proteins of precartilage and cartilage cells electrophoresed on a 12.5% $NaDodSO_4$–polyacrylamide slab gel. Nuclei were prepared from freshly explanted apical mesoderm of 5-day-old embryonic chick wing buds and from 7-day-old embryonic limb and vertebral cartilages. The use of the 5-day-old wing tips as our source of precartilage cells eliminates contamination by the myogenic cell subpopulation found in early limb buds (Newman, 1977; Newman et al., 1981). For convenience, we have used 7-day-old chick embryos as our source of definitive cartilage cells instead of the 3-day-old cultured wing tips originally described (Newman et al., 1976). Chromatin of the in vivo differentiated cartilage differs somewhat more extensively from the progenitor cell chromatin than does that of the newly differentiated cartilage grown in vitro. However, the loss of abundant components with molecular weights of approximately 125 000 (PCP 125) and 35 500 (PCP 35.5) remains the most prominent difference between the precartilage and cartilage chromatin preparations (Figure 1).

Because the abundant 35.5 component of precartilage nuclei partially overlaps with a less abundant component of similar mobility on one-dimensional gels of both precartilage and cartilage chromatin, we performed two-dimensional gel electrophoresis (O'Farrell, 1975) on precartilage and cartilage chromatin proteins (not shown). We find that one abundant, neutral precartilage nonhistone protein of M_r 35 500 is indeed undetectable, and a second basic one (pI $\sim$ 8.5) is either diminished or absent in cartilage chromatin.

In addition to PCP 125 and PCP 35.5, a number of highly reproducible qualitative and quantitative differences are observed in the nonhistone protein complements of precartilage and cartilage nuclei, against an apparently constant background of other nonhistones.

Tight Binding of PCP 35.5 to Chromatin DNA. Because our goal was to determine the localization of specific nonhistone proteins relative to nuclease-sensitive chromatin domains, we were interested in identifying nonhistones of the tight DNA-binding class (Pederson & Bhorjee, 1975; Bekhor & Mirell, 1979), as these would constitute the subpopulation least likely to redistribute from their native sites during mild nuclease cleavage of chromatin (Pederson & Bhorjee, 1975). We found that a modification of the procedure of Pederson & Bhorjee (1975) in which we dissolved precartilage chromatin in 2.5 M NaCl and 3.2 M urea and centrifuged the solution under conditions in which virtually all the DNA and tightly complexed proteins were pelleted yielded a pellet fraction highly enriched in PCP 35.5 and several other proteins (Figure 2). The remainder of the precartilage nonhistones were found in the supernatant fraction with less than 2% of the total chromatin DNA.

Selective Release of Nuclear Nonhistone Proteins by Limited Digestion with DNase I. Precartilage and cartilage nuclei were treated with DNase I following the procedure of Weintraub & Groudine (1976). The time course of digestion for each population of nuclei exhibited biphasic kinetics, with the rapidly solubilized component of the precartilage chromatin DNA comprising 40–50% of the total and that of cartilage chromatin DNA comprising 20–30% of the total (Figure 3). The percent solubilization at which the rate of digestion slowed down was reproducible within the given limits in at least six independent determinations for each cell type.

Figure 4 shows the pattern of proteins released into the supernatant after each nuclear population was digested for enough time to solubilize the rapidly attacked chromatin DNA. The supernatant fractions clearly contain a limited subset of nuclear proteins which are partly overlapping and partly disjoint in the two cell types.

Because reaggregation of digestion-released proteins has been reported (Billing & Bonner, 1972), it is important to note that all digestions were stopped by making the reaction mixtures 20 mM EDTA and 3.3 M urea to minimize this effect. Indeed, virtually all release of protein was dependent on this step.

In experimental controls, nuclei incubated under identical conditions, but in the absence of DNase I, and treated with the same EDTA–urea stopping buffer lose essentially no proteins detectable on gels. Thus the observed fractionation of chromatin proteins is achieved by specific DNase I attack and is not due to endogenous nucleolytic or proteolytic cleavage.

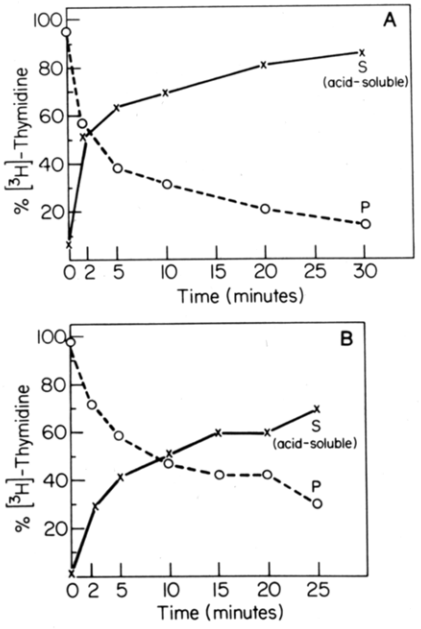

FIGURE 3: Kinetics of DNase I digestion of precartilage and cartilage chromatin. Cells of each type were labeled in ovo with [^{3}H]thymidine and their nuclei digested with DNase I for times indicated. Percent of [^{3}H]thymidine in each fraction is based on total radioactivity (cpm) calculated individually for each time point. P is the pellet of acid insoluble material; S is the acid-soluble supernatant. (A) Digestion of precartilage nuclei; (B) digestion of cartilage nuclei.

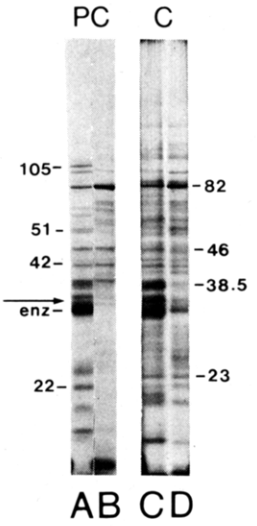

FIGURE 4: Proteins released from precartilage and cartilage chromatin by digestion with DNase I or with a mixture of ribonucleases. Released proteins were precipitated with acid as described under Materials and Methods and electrophoresed on $NaDodSO_4$–12.5% polyacrylamide slab gels. All slots contain supernatants from 5×10^6 nuclei. (A) Proteins released from precartilage chromatin by DNase I; (B) proteins released from precartilage chromatin by RNases A and T_1; (C) proteins released from cartilage chromatin by DNase I; (D) proteins released from cartilage chromatin by RNases A and T_1. PC, precartilage; C, cartilage; enz, DNase I; arrow, position of PCP 35.5 (absent on this gel). Numbers represent $M_r/10^3$.

It is therefore of interest that the precartilage chromatin proteins PCP 125 and PCP 35.5 are not detected in the supernatant generated by DNase I digestion of precartilage nuclei but remain in the residual pellet. This result suggests that these proteins are not directly associated with chromatin DNA that is highly susceptible to attack by DNase I.

Selective Release of Nuclear Proteins by RNases. Some, but not all, of the nonhistone proteins released with DNase I sensitive chromatin DNA would be expected to have an

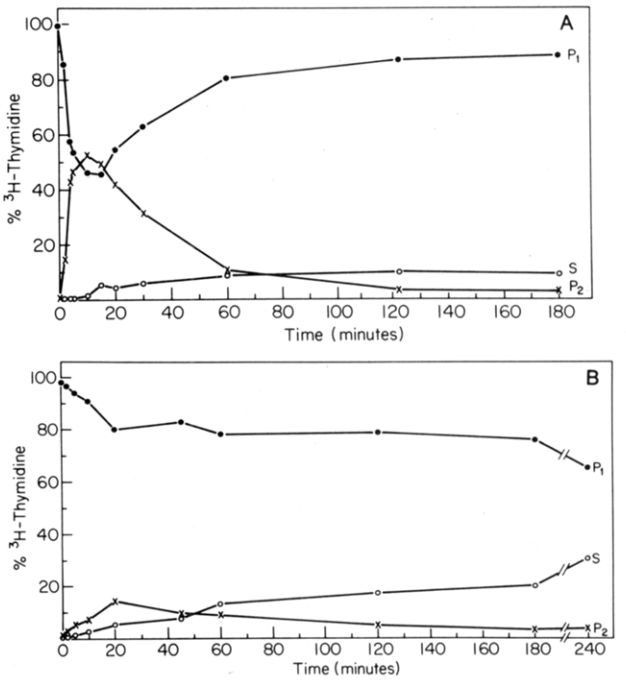

FIGURE 5: Kinetics of digestion of precartilage and cartilage chromatin with DNase II. Precartilage and cartilage cells were labeled in ovo with [^{3}H]thymidine and fractionated into P1 (undigested), P2 (released Mg^{2+} insoluble), and S (released Mg^{2+} soluble) fractions after various times of treatment with DNase II as described under Materials and Methods. Values for each fraction were calculated as percentages of total radioactivity sampled at respective time points. The S fraction was largely acid soluble throughout the time course of digestion for both chromatin types. Note reaggregation of chromatin DNA with the P1 fraction during the time course of digestions. (A) Digestion of precartilage chromatin; (B) digestion of cartilage chromatin.

RNA-associated function, since such DNA has been shown to be template competent in all systems in which this correlation has been investigated (Weintraub & Groudine, 1976; Levy & Dixon, 1977; Bellard et al., 1977; Mayfield et al., 1978). We therefore extensively treated nuclei from precartilage or cartilage cells with a mixture of ribonucleases A and T_1 and monitored released proteins. For each cell type, the population of proteins released by the ribonucleases extensively overlaps but is not totally coincident with the population released by DNase I (Figure 4). Similarly to DNase I, the RNases release no PCP 125 or PCP 35.5 from precartilage chromatin.

Selective Release of Chromatin Fractions by Progressive Digestion with DNase II. The endonuclease DNase II has been used by several groups to fractionate chromatin into functionally distinct subfractions (Gottesfeld et al., 1974, 1975; Goldsmith, 1981). Under similar conditions to those used for other cell types by previous investigators (Gottesfeld et al., 1974, 1975), we have found that the DNA in both precartilage and cartilage chromatin is partitioned by DNase II almost exclusively into the P1 (not attacked by the enzyme) and P2 (released by the enzyme, but insoluble in 5 mM Mg^{2+}) fractions (Figure 5). In these experiments we have deliberately not controlled for reaggregation of chromatin during digestion, which serves mainly to enhance P1 at the expense of P2 and is particularly evident in the case of precartilage (Figures 5 and 6). We have also made no a priori assumptions concerning the efficacy of DNase II in fractionating template active from template inactive chromatin. The primary utility of this fractionation protocol in our hands is that after release of a precartilage P2, and reassociation of most of this fraction with the P1, we consistently obtain a small "limit" P2 fraction highly

enriched in PCP 35.5 and PCP 125. These proteins are present in the P2 from the earliest stages of digestion and remain with this fraction despite its progressive loss of other proteins and a large amount of DNA during this process. The enrichment of the limit P2 fraction in DNase I sensitive DNA sequences (see below) indicates that PCP 35.5 (and possibly PCP 125) is concentrated near chromatin domains of potential regulatory significance.

The percentages of DNA found in each of the DNase II generated fractions for each cell type at progressive stages of digestion are shown in Figure 5. It can be seen that the Mg^{2+}-soluble fraction, S, is never a significant proportion of released chromatin in either cell type until stages when extensive reaggregation of P2 into the P1 fraction has taken place. Furthermore, this S fraction is always largely acid soluble, in contrast to the Mg^{2+}-soluble fraction in the systems examined by Gottesfeld and co-workers.

The protein compositions of the various DNase II generated fractions are shown in Figure 6 for both cell types. The entire protein content of each subfraction has been loaded on the gel for each time point so that relative enrichments can be determined from these gels. Although evidence of chromatin reaggregation seen in the DNA data of Figure 5 is also seen in these gels, the patterns of proteins found in the S and P2 fractions are relatively stable throughout the course of the digestions.

It can be seen from Figure 6 that the nonhistone protein complements of the two DNase II released fractions are nearly identical within each cell type. In addition, each cell type's P2 also contains stoichiometric amounts of all histones.

We also note that the proteins released into the S and P2 by DNase II are virtually identical with those released by DNase I from each nuclear type (compare Figure 6 with Figure 4). The main exceptions to this observation are the release into the P2 fraction of precartilage chromatin of large amounts of PCP 35.5 and of PCP 125. These proteins are not released from precartilage chromatin by DNase I (Figure 4).

If digestion of precartilage chromatin with DNase II is allowed to continue for long periods, most of the P2 DNA and its associated proteins (including, occasionally, H1) are lost to other fractions. What remains in the limit P2 is 5–9% of the nuclear DNA complexed with histones and a small number of nonhistones, the most prominent of which are PCP 35.5 and PCP 125 (about half the cells' complement of each).

Relationship between Precartilage Limit P2 Fraction and DNase I Sensitive DNA Sequences. In order to determine the relationship between the precartilage DNA sequences susceptible to attack by DNase I and those released by DNase II, we made use of precartilage nuclei radioactively labeled in their DNase I sensitive regions, prepared by the nick translation procedure described by Cedar and co-workers (Levitt et al., 1979; Gazit et al., 1980). Figure 7A shows the DNase I dependence of incorporation of radioactivity into precartilage nuclei in the nick translation reaction. In Figure 7B the results are shown of parallel digestions by DNase I of nuclear preparations, one of which was uniformly labeled in DNA in ovo and one of which was labeled by nick translation. It can be seen that 85% of the nick-translated radioactivity is removed from the nuclei during the early, rapid phase of digestion by DNase I when 25–50% of the bulk DNA is solubilized.

Under the assumption that the DNase I sensitive regions of precartilage chromatin are uniformly labeled in the nick translation reaction, we can calculate the extent to which any fraction of chromatin, released by any procedure, is enriched

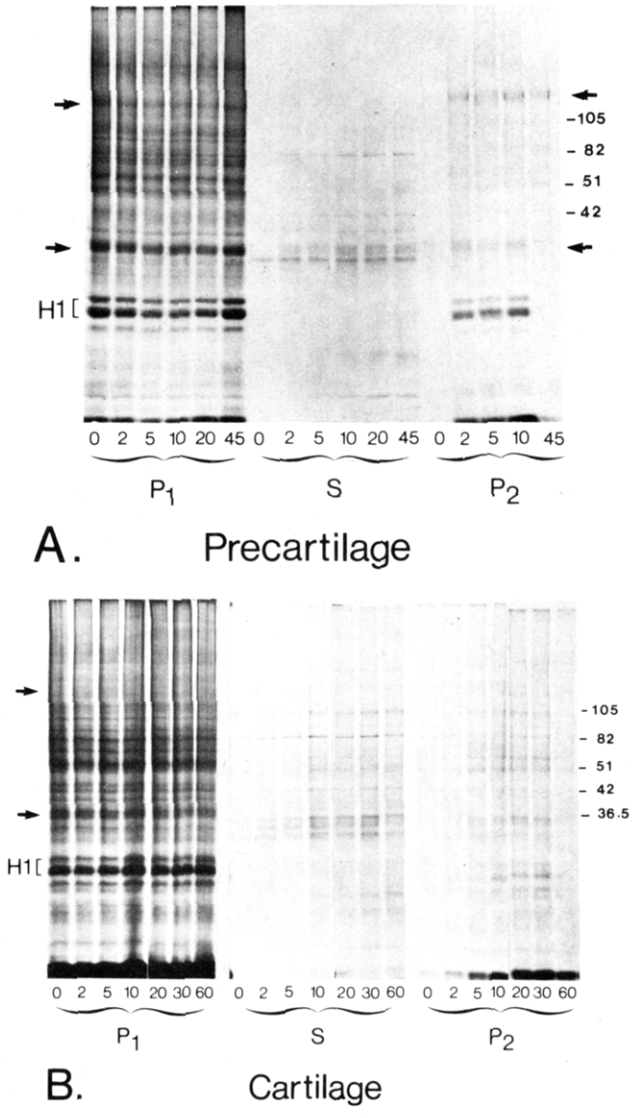

FIGURE 6: Proteins comprising DNase II generated fractions at successive times of digestion of precartilage and cartilage chromatin with DNase II. Fractions were prepared from precartilage and cartilage nuclei as described for Figure 5, and proteins were electrophoresed on $NaDodSO_4$–12.5% polyacrylamide slab gels. (A) Proteins comprising precartilage DNase II generated chromatin fractions; (B) proteins comprising cartilage DNase II generated chromatin fractions. In all cases the entire P1, S, and P2 fractions from a given time point (3×10^6 nuclei) were dissolved in sample buffer and loaded on the gel. Arrows in (A) indicate PCP 35.5 and PCP 125; arrows in (B) indicate positions of PCP 35.5 and PCP 125, which are absent in cartilage chromatin. Numbers represent $M_r/10^3$.

in DNase I sensitive DNA, if we measure the proportion of both in ovo incorporated and nick translation incorporated radioactivity present in that fraction. The percent enrichment, E, is calculated from

$$E = \left(\frac{\%\ \text{nick-translated label}}{\%\ \text{in ovo label}} \Big/ 2.5 \right) \times 100$$

where the factor 2.5 corresponds to the information in Figure 7B that a maximum of 85% of the nick-translated radioactivity is located in DNase I sensitive regions and that this radioactivity is concentrated in 34% of total chromatin DNA.

On the basis of this formula we have calculated the degree of enrichment in DNase I sensitive DNA of the P2 fractions generated by DNase II at progressive times of digestion. The data shown in Figure 8 were generated from matched preparations of in ovo labeled and nick-translated precartilage

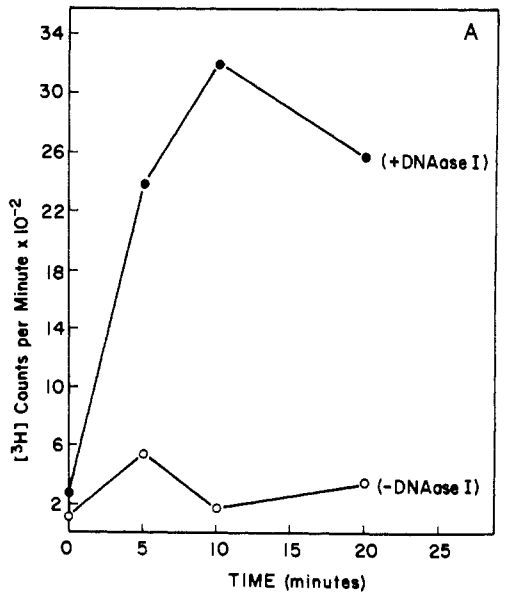

FIGURE 7: Nick translation of precartilage nuclei and sensitivity of incorporated radioactivity to DNase I. (A) Nick translation was performed as described under Materials and Methods with and without the addition of DNase I to determine dependence on this enzyme of incorporation of [^{3}H]TTP. Incubation with *E. coli* DNA polymerase I was for various amounts of time to determine the point of maximal DNase I dependent incorporation. (B) Nick translation was performed under standard conditions (precartilage nuclei pretreated with DNase I, incubated with DNA polymerase for 10 min). A matched preparation of nuclei was labeled uniformly in ovo with [^{3}H]thymidine. Nuclear preparations were digested in parallel with DNase I for successive times. A comparison of the two graphs indicates the concentration in bulk chromatin of nick translation incorporated radioactivity.

nuclei, digested in parallel with the same lot of DNase II; however, the results for each digestion essentially reproduced at least two other independent determinations.

As can be seen in Figure 8, the P2 fraction becomes progressively more enriched in DNase I sensitive DNA as its absolute mass is decreased by reaggregation of early released DNA with the P1 fraction (see Figure 5A). The 45-min P2 fraction is seen to be about 90% enriched in DNase I sensitive DNA.

We were concerned that the various DNase II generated fractions might have contained unequal proportions of the roughly 15% of nick-translated radioactivity that is introduced nonspecifically and is resistant to redigestion by DNase I (see Figure 7). This would have altered our estimates of enrichment in DNase I sensitive DNA for the P2 fractions reported

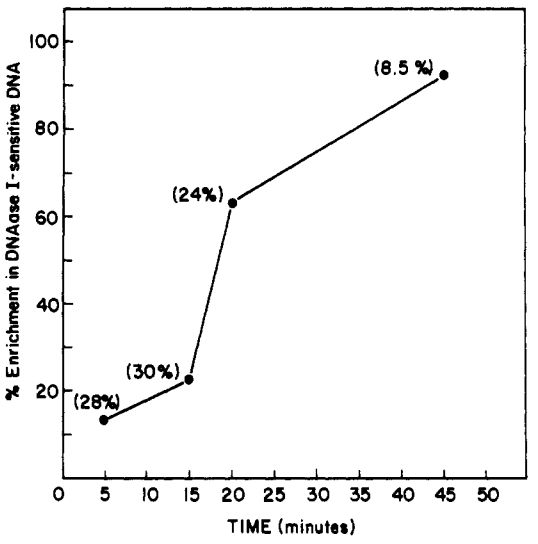

FIGURE 8: Enrichment in DNase I sensitive DNA sequences of precartilage chromatin P2 fractions generated by successive times of treatment with DNase II. Matched preparations of nuclei were labeled in ovo and nick translated as in Figure 7B. Each nuclear preparation was treated with DNase II for increasing times, and P1, P2, and S fractions were prepared. Enrichment in DNase I sensitive sequences for each fraction was calculated as described in the text. Enrichment of P2 fractions is shown. Numbers next to each point indicate percentage of total chromatin DNA in P2 fractions at times indicated.

in Figure 8. Therefore, we extensively DNase I digested the P1 and P2 fractions generated from a DNase II digestion of nick-translated nuclei after 45 min. About 15% of the radioactivity of each fraction remained resistant to DNase I, indicating that the resistant material is proportionately distributed.

We conclude that the precartilage limit P2 fraction, which contains 5–9% of precartilage chromatin DNA along with a subset of nonhistone proteins, including abundant amounts of PCP 125 and PCP 35.5, is approximately 90% enriched in DNase I sensitive DNA sequences.

Association of PCP 35.5 and PCP 125 with DNase I Resistant DNA Sequences Adjacent to DNase I Sensitive Sequences in Precartilage Chromatin. Because PCP 35.5 and PCP 125 can be isolated from precartilage chromatin as part of a domain that consists mostly of DNase I sensitive sequences, but are not themselves dislodged from chromatin by DNase I, there is a strong possibility that they interact with a chromatin structure flanking template-activated sequences (Stalder et al., 1980b; Weintraub et al., 1981). Removal of the DNase I sensitive chromatin from the limit P2 fraction should define a small chromatin subfraction of possible regulatory significance. When a 45-min precartilage P2 fraction is redigested with DNase I, approximately 12% of the DNA comprising this fraction (which is itself 8% of the total DNA; see Figure 8) resists solubilization. We find that histones, most of the nonhistones, all of the PCP 125, and most of the PCP 35.5 contained in the original P2 fractions remain complexed with the resistant 1% of precartilage DNA. Initial studies of this trimmed fraction show the DNA to consist largely of a collection of kilobase-sized fragments (data not shown).

It can be concluded that large amounts of PCP 35.5 and PCP 125 and smaller amounts of other precartilage chromatin proteins are contained in a DNase I resistant chromatin structure closely associated with DNase I sensitive chromatin domains.

Discussion

The foregoing studies have identified several nonhistone

proteins that are concentrated in specific chromatin subfractions of embryonic chick precartilage and cartilage nuclei. The precise manner in which certain of these proteins are distributed is suggestive of roles analogous to previously described nonhistones. Specifically, the M_r 38 500 component (released by DNase I but not by the RNases in both cell types; Figure 4) could play a general role in the sensitive chromatin state in this lineage, analogous to the *Drosophila* nonhistone studied by Elgin and her co-workers (Mayfield et al., 1978). In contrast, the precartilage M_r 22000 component (cell type specific; Figure 1; released by DNase I but not by the RNases; Figure 4) could play a role with respect to the sensitive conformation in sequences of the precursor cell not in the same state in the terminal cell. In the absence of probes for particular DNA sequences, we can make no inference concerning specific genes that may be associated with these proteins. However, because DNase I sensitivity of chromatin DNA has been correlated with template activation in a wide variety of tissues (Weintraub & Groudine, 1976; Garel & Axel, 1976; Levy & Dixon, 1977; Bellard et al., 1977; Mayfield et al., 1978), these proteins are candidates for regulatory molecules in their respective cell types.

It is conceivable that PCP 35.5 and PCP 125 are also in the DNase I sensitive regions of precartilage chromatin but rapidly reaggregate with bulk chromatin upon release by the nuclease; however, we think this is unlikely. In the first place, DNase I digested nuclei were centrifuged in the presence of 20 mM EDTA and 3.3 M urea, a treatment in excess of that found to eliminate reaggregation in an analogous study (Billing & Bonner, 1972). Furthermore, our DNase II data demonstrate that these two proteins remain unaggregated with bulk chromatin under conditions in which all other released nonhistones reaggregate with the bulk fraction (Figure 6A). The very strong affinity between PCP 35.5 and DNA (Figure 2) virtually ensures that it coisolates with its native binding sequences after mild DNase II digestion, but we have no similar assurance regarding PCP 125, which, while it also may be a DNA-binding protein, does not remain bound to DNA under our tight-binding assay conditions (Figure 2). We are therefore more confident, at the present time, of the nuclear proximity of PCP 35.5 to at least 25% of the DNase I sensitive DNA sequences in precartilage chromatin than we are of the localization of PCP 125. Our results on cartilage chromatin have not revealed any abundant nonhistone that plays a role in that cell type analogous to PCP 35.5, implying that we may be observing a phenomenon peculiar to development.

It is notable that the two major proteins that remain associated with the limit P2 fraction of precartilage are also the most prominent markers distinguishing precartilage from cartilage chromatin (Newman et al., 1976; Perle & Newman, 1980; Figure 1). Furthermore, these two proteins are either altered (PCP 125) or reduced in amount (PCP 35.5) in the precartilage mesenchyme of *talpid*[2] mutants, whose pattern of cartilage differentiation is highly perturbed (Perle & Newman, 1980). Finally, PCP 35.5 is phosphorylated during cartilage differentiation in vitro and is the only component detectably phosphorylated in response to physiological levels of cyclic AMP in isolated precartilage nuclei (S. A. Newman and C. M. Leonard, unpublished results). Taken together with the present findings, these results are suggestive of regulatory roles during cartilage differentiation for PCP 35.5 and PCP 125, possibly along the lines of the "domain attachment points" hypothesized by Igo-Kemenes & Zachau (1977) [see also Benyajati & Worcel (1976) and Paulson & Laemmli (1977)].

We do not know whether the DNase I sensitive regions associated with the PCP-containing structure represent sequences which are transcribed in the precartilage cell. Alternatively, they may represent a "preactivation chromosomal state" of sequences to be expressed in the cartilage cell, analogous to that found for adult globin genes in primitive series erythrocytes by Stalder et al. (1980a). If this were the case, removal of PCP 35.5 (and possibly PCP 125) during development might serve to unblock these capacitated sequences and make them available for transcription. Experiments using probes specific for genes differentially active precartilage and cartilage cells are clearly necessary to determine the nature of the sensitive sequences associated with PCP 125 and PCP 35.5.

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Effects of pH, Ionic Strength, and Temperature on Activation by Calmodulin and Catalytic Activity of Myosin Light Chain Kinase†

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ABSTRACT: The reversible association of Ca_4^{2+}-calmodulin with the inactive catalytic subunit of myosin light chain kinase results in the formation of the catalytically active holoenzyme complex [Blumenthal, D. K., & Stull, J. T. (1980) *Biochemistry 19*, 5608-5614]. The present study was undertaken in order to determine the effects of pH, temperature, and ionic strength on the processes of activation and catalysis. The catalytic activity of myosin light chain kinase, when fully activated by calmodulin, exhibited a broad pH optimum (>90% of maximal activity from pH 6.5 to pH 9.0), showed only a slight inhibition by moderate ionic strengths (<20% inhibition at $\mu = 0.22$), and displayed a marked temperature dependence ($Q_{10} \simeq 2$; $E_a = 10.4$ kcal mol^{-1}). Thermodynamic parameters calculated from Arrhenius plots indicate that the Gibb's energy barrier associated with the rate-limiting step of catalysis is primarily enthalpic. The process of kinase activation by calmodulin had a narrower pH optimum (pH 6.0-7.5) than did catalytic activity, was markedly inhibited by increasing ionic strength (>70% inhibition at $\mu = 0.22$), and exhibited nonlinear van't Hoff plots. Between 10 and 20 °C, activation was primarily entropically driven ($\Delta S° \simeq 40$ cal mol^{-1} deg^{-1}; $\Delta H° = -900$ cal mol^{-1}), but between 20 and 30 °C, enthalpic factors predominated in driving the activation process ($\Delta S° \simeq 10$ cal mol^{-1} deg^{-1}; $\Delta H° = -9980$ cal mol^{-1}). The apparent change in heat capacity (ΔC_p) accompanying activation was estimated to be -910 cal mol^{-1} deg^{-1}. On the basis of these data we propose that although hydrophobic interactions between calmodulin and the kinase are necessary for the activation of the enzyme, other types of interactions such as hydrogen bonding, ionic, and van der Waals interactions also make significant and probably obligatory contributions to the activation process.

Calmodulin is known to regulate a number of enzymes and cellular processes in a Ca^{2+}-dependent manner [for reviews, see Wolff & Brostrom (1979), Klee et al. (1980), Cheung (1980), Means & Dedman (1980), and Wang & Waisman (1979)]. Myosin light chain kinase is one of the several enzymes whose activity is completely dependent on the presence of Ca^{2+} and calmodulin. This enzyme is responsible for catalyzing the phosphorylation of a specific subunit of myosin, known as the phosphorylatable or P light chain (Frearson & Perry, 1975). The enzyme and its substrate are present in nonmuscle as well as muscle tissue. The phosphorylation reaction may play an important role in the regulation of contraction in smooth and skeletal muscles (Stull, 1980; Stull et al., 1980), as well as in modulating cellular function in certain nonmuscle tissues (Adelstein, 1978; Salisbury et al., 1980).

The details of the interaction of calmodulin with the many proteins it regulates are not well understood. Previous studies in our laboratory were concerned with determining the mechanism of activation of myosin light chain kinase (Blumenthal & Stull, 1980). The purpose of this investigation was to extend our previous studies and to determine the effects of pH, temperature, various salts, and ionic strength on the activation and catalytic activity of myosin light chain kinase. From analysis of these results it is possible to obtain information regarding the factors that play important roles in the regulation of myosin light chain kinase activity. Because calmodulin is highly conserved throughout eukaryotic evolution (Jamieson et al., 1980), the general features of the interaction of calmodulin with myosin light chain kinase may be applicable to other calmodulin-dependent processes.

Materials and Methods

Chemicals were obtained from Sigma and were of analytical grade or better. [γ-^{32}P]ATP[1] was prepared by the method

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; Mops, 4-morpholinepropanesulfonic acid; Hepps, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; ATP, adenosine 5'-triphosphate; NMR, nuclear magnetic resonance; SEM, standard error of the mean.

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