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Nuclear Localization of Type II cAMP-Dependent Protein Kinase during Limb Cartilage Differentiation Is Associated with a Novel Developmentally Regulated A-Kinase Anchoring Protein

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Differentiation of chicken limb cartilage is accompanied by a rise in intracellular cyclic AMP, an inducer of cartilage-specific gene expression. A basic ~35-kDa protein, designated p35, is the major nuclear substrate for cAMP-dependent protein kinase (PKA) during this process. Here we show that whereas both precartilage and cartilage nuclei contain p35, only precartilage nuclei contain PKA. The phosphorylation of p35 in isolated cell fractions was used as an index of changes in the cellular comparmentalization of components of PKA during chondrogenesis. Both the catalytic subunit and type II regulatory subunit (RII) of PKA were present in the precartilage nuclear fraction, but were undetectable or present in only trace amounts in the cartilage nuclear fraction. Furthermore, a novel ~150-kDa A-kinase anchoring protein (AKAP), which binds to RII, was detected in the nuclear matrix of precartilage nuclei but, like RII, was virtually absent in the nuclei of fully differentiated cartilage cells. In limb mesenchymal cells undergoing chondrogenesis in culture a corresponding set of changes occurred: cartilage differentiation was accompanied by a marked reduction in the amounts of both nuclear RII and nAKAP150. These observations indicate that type II PKA holoenzyme is imported into the mesenchymal cell nucleus prior to chondrogenesis, an event that appears to depend on the the activity of the developmentally regulated nAKAP150.

INTRODUCTION

Cartilage development in the mesenchyme of the vertebrate limb is an advantageous system for studying aspects of the spatiotemporal regulation of cell differentiation both *in vivo* and *in vitro* (Newman, 1988, 1993). An important aspect of this process is the intracellular transduction of signals initiated by extracellular events. During the crucial cellular condensation process in which precartilage mesenchymal cells interact closely with one another prior to synthesizing cartilage matrix, there is a transient increase in cellular cyclic AMP content (Ho *et al.*, 1982; Biddulph *et al.*, 1988). Because agents which elevate cellular cAMP levels *in vitro* stimulate chondrogenesis (Kosher *et al.*, 1979; Solursh *et al.*, 1981) by a process which includes marked increases in the expression of genes that specify cartilage-specific proteins (Kosher *et al.*, 1986a,b), it has been proposed that limb chondrogenesis *in vivo* is regulated by cell–cell interactions that elevate cAMP (Newman, 1988).

The components and mechanisms of cAMP mediation of new gene expression during chondrogenesis are not well understood. Previous studies (Leonard and Newman, 1987) demonstrated that a nuclear protein of M_r ~35 kDa (p35),[4]

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[4] Abbreviations used: p35, ~35-kDa basic phosphoprotein of precartilage mesenchymal cells; PKA, cAMP-dependent protein ki-

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which undergoes phosphorylation during cartilage differentiation in cultured limb mesenchymal cells, became hyperphosphorylated when chondrogenesis was stimulated by treatment with dibutyryl cAMP. In isolated precartilage nuclei the phosphorylation of p35 was entirely dependent on cAMP (Leonard and Newman, 1987). These results, along with the finding that p35 coisolated with a chromatin fraction enriched in template active DNA sequences (Leonard and Newman, 1987; Perle *et al.,* 1982), suggested that this protein had a role in transducing the cAMP signal in precartilage cell nuclei. In the present study we have used the capacity of p35 to undergo phosphorylation in isolated nuclear and extranuclear fractions of limb precartilage and cartilage cells as a tool to investigate the redistribution of the components of the cAMP-dependent protein kinase (PKA) during chondrogenesis. Our results show that the PKA holoenzyme is localized in the precartilage nucleus prior to overt chondrogenesis, but is no longer present in the cell nucleus after cartilage differentiation has occurred.

We have also considered possible mechanisms by which the nuclear localization of PKA is regulated during chondrogenesis. The PKA holoenzyme contains a regulatory subunit dimer and two catalytic subunits. The two classes of PKA, type I and type II, have a common catalytic subunit but differ with respect to their regulatory subunits, RI and RII. Previous work has provided evidence that subcellular localization of PKA may be directed by the regulatory subunit (Scott, 1991). RI isoforms are primarily cytoplasmic. In contrast, certain tissues contain up to 75% of their RII isoforms in particulate form, associated with either the plasma membrane, cytoskeletal components, secretory granules, or the nuclear membrane (Scott, 1991). Type II kinase localization is thought to be mediated by association of RII with specific anchoring proteins, referred to as A-kinase anchoring proteins (AKAPs) (Hirsch *et al.,* 1992; Carr and Scott, 1992; Carr *et al.,* 1992a,b; 1993). Presumably, PKA anchoring influences which substrates are most accessible to the catalytic subunit upon elevation of cAMP. Accordingly, this process may adapt tissues for cell type-specific cAMP responsive events (Scott, 1991).

We report here that precartilage nuclei contain an ~150-kDa AKAP, designated nAKAP150, which is absent from the nuclei of definitive chondrocytes. Although AKAPs typically have restricted cell type distributions (Scott *et al.,* 1990), nAKAP150 represents the first such protein determined to be developmentally regulated. We suggest that nAKAP150 may mediate the localization of PKA in the nuclei of precartilage mesenchymal cells during the period when the rapidity and accuracy of these cells' response to spatiotemporally distributed signals is most critical.

nase; RI, RII, type I and type II regulatory subunits of PKA; PKI, heat stable inhibitor of PKA; LDH, lactate dehydrogenase; NEPHGE, nonequilibrium pH gradient gel electrophoresis; 8-azido cAMP, 8-azidoadenosine-3′,5′-cyclic monophosphate; AKAP, A-kinase anchoring protein; nAKAP150, ~150-kDa nuclear AKAP.

MATERIALS AND METHODS

Cell fractionation. Limb mesenchyme was isolated from wing bud tips of White Leghorn chicken embryos (Avian Services, Inc., Frenchtown, NJ) at Hamburger–Hamilton stage 24–25 (Hamburger and Hamilton, 1951) as previous described (Newman *et al.,* 1976; Perle *et al.,* 1982). This tissue is a virtually pure population of precartilage mesenchymal cells and is devoid of premuscle mesenchyme (Newman, 1977; Newman *et al.,* 1981). Embryonic limb cartilage was separated from muscle and connective tissue by agitation of whole wings from 7-day chick embryos (stage 29–30; Hamburger and Hamilton, 1951) in ice-cold distilled H_2O with a Vortex-Genie mixer, and sedimentation at unit gravity as described (Linsenmayer, 1974; Perle *et al.,* 1982). Nuclei were prepared from each tissue by homogenization in nuclear preparation buffer (NPB: 10 m*M* NaCl, 3 m*M* $CaCl_2$, 0.25 *M* sucrose, 10 m*M* Tris, pH 7.4, 0.5% NP-40), filtration though Nytex (20-μm mesh, Tetko Inc., Briarcliff Manor, NY), and centrifugation as described (Perle *et al.,* 1982; Leonard and Newman, 1987). Extranuclear supernatants (including cytosol and extracellular matrix) from precartilage mesenchyme and cartilage were dialyzed against one-tenth strength buffer A (buffer A: 30 m*M* Tris–HCl, pH 7.4, 30 m*M* NaCl, 9 m*M* $MgCl_2$), lyophilized, and resuspended in water to one-tenth the original volume. In some experiments a mixture of protease inhibitors (2 μg/ml leupeptin; 1 μg/ml pepstatin; 1 m*M* benzamidine; 50 μg/ml phenylmethylsulfonyl fluoride (PMSF)) was included during preparation of the tissue fractions. The results reported were essentially unaffected by these additions. Nuclear and extranuclear fractions were stored at −80°C until needed.

The efficacy of the nuclear–extranuclear fractionation procedure was verified by use of cytosolic and nuclear markers. Lactate dehydrogenase (LDH) activity, a cytosolic marker, was measured spectrophotometrically by monitoring the rate of pyruvate-dependent oxidation of NADH (Wroblewski and LaDue, 1955). One unit of enzyme was defined as that activity causing a decrease in optical density of 0.001 per minute in a standard 1-ml reaction (Wroblewski and LaDue, 1955). Immunoblotting (see below) using antibodies against the nuclear markers CREB (a gift of Dr. Edward Ziff, NYU School of Medicine) and fos (a gift of Dr. Rodrigo Bravo, Bristol-Myers Squibb Research Institute) was also used to validate the fractionation procedure.

In some cases nuclei were prepared by a nonaqueous procedure (Lynch *et al.,* 1975; Miwa *et al.,* 1990) to control for possible redistribution of soluble components during isolation. Limb bud precartilage mesenchyme was quick frozen in liquid nitrogen immediately upon dissection and lyophilized for 3 days. The freeze-dried tissue was then transferred to a desiccator containing P_2O_5, which was immediately evacuated. After 3 hr the samples were powdered with a mortar and pestle and passed through a 110 mesh wire screen. The powder (5 mg) was then homogenized for 40 sec, at 10-sec intervals, in 0.4 ml glycerol using a Polytron P-10 homogenizer (Brinkmann Instruments). Examination of the homogenate by phase contrast microscopy confirmed that it contained no fragments larger than nuclei. Each 0.2-ml aliquot of the homogenate was layered over 3 ml glycerol and centrifuged for 18 hr at 30,000 rpm at 4°C in the Spinco SW50.1 rotor. The nuclei and nuclear fragments were recovered as the pellet. The remaining material was recovered by precipitation after dilution of the glycerol with aqueous trichloroacetic acid, and designated the extranuclear fraction.

In each experiment comparing nuclear and extranuclear fractions

of precartilage and cartilage cells, material from equivalent numbers of cells was used. Nuclear and extranuclear proteins were quantitated using the Bio-Rad (Richmond, CA) protein assay kit, based on the Bradford dye binding procedure (Bradford, 1976), with bovine serum albumin as standard.

Protein phosphorylation and electrophoresis. Phosphorylation of nuclear and extranuclear proteins from precartilage and cartilage cells was carried out at 30°C in buffer A containing 5–25 μM [γ-^{32}P]ATP (Amersham or NEN, adjusted to 100 cpm/fmole) for 10 min, in the absence or presence of 10 μM cAMP (Sigma). Where indicated, PKI(11-24) (Scott *et al.*, 1986), an active fragment of the heat-stable protein kinase inhibitor (Walsh *et al.*, 1971), was used at 100 μM, and the catalytic subunit of PKA (Sigma) was used at 16 units per 40-μl reaction. Phosphorylation reactions were terminated by the addition of Laemmli sample buffer (Laemmli, 1970), or by centrifugation at 4°C. Samples were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) using either 12.5% resolving gels (acrylamide/bisacrylamide, 120/1; Blattler *et al.*, 1972), or 5–15% gradient gels (acrylamide/bisacrylamide, 32/1). In some experiments, phosphorylated nuclei were treated with 30 μg DNase/ml for 30 min as described (Leonard and Newman, 1987), and subjected to two-dimensional nonequilibrium pH gradient gel electrophoresis (NEPHGE) (O'Farrell *et al.*, 1977). Electrophoresis in the first dimension was performed at 600 V·hr with pH 3–10 ampholyte (BioRad), and in the second dimension using a 12.5% resolving gel. Autoradiography was performed by exposure of X-OMAT AR film (Kodak) to dried gels at −80°C. In each experiment in which a comparison between cell types is made, the specific activity of the radioisotope and exposure time of the autoradiograms were identical. Tryptic mapping, using two-dimensional thin layer chromatography (Lerea, 1992), was performed to verify the identity of NEPHGE phosphoprotein spots from different tissue preparations.

Photoaffinity labeling. Cyclic AMP binding proteins were identified by using [^{32}P]8-azidoadenosine-3′,5′-cyclic monophosphate ([^{32}P]8-azido-cAMP) as a photoaffinity probe (Walter *et al.*, 1977). Each reaction (40 μl) contained nuclei or extranuclear extract from equivalent numbers of cells, and 1 μM [^{32}P]8-azido-cAMP (50 Ci/mmol, ICN) with or without 100 μM nonradioactive cAMP as competitor. Samples were incubated in the dark at 4°C for 1 hr and then irradiated for 1 min at 254 nm with a Mineralight UVG-54 lamp at a distance of 8 cm, at room temperature.

Immunoblot analysis. A polyclonal antibody prepared against a synthetic peptide corresponding to C-terminal amino acid residues of the human PKA catalytic subunit was obtained from UBI (Lake Placid, NY). Polyclonal anti-murine RII antibody and recombinant RII were as previously described (Carr *et al.*, 1992a,b). Nuclear and extranuclear proteins from precartilage and cartilage cells were electrophoresed on 12.5% SDS–polyacrylamide gels, followed by transfer to nitrocellulose by electroblotting (Bio-Rad Trans-Blot electrophoretic transfer cell). The nitrocellulose filter was incubated with anti-catalytic subunit antibody (1/500 dilution) or anti-murine RII antibody (1/1000 dilution), followed by HRP-goat anti-rabbit antibody, which allowed detection by the enhanced chemiluminescence (ECL) assay according to the directions of the manufacturer (Amersham), or by ^{125}I-labeled goat anti-rabbit antiserum (Gizang-Ginsberg and Ziff, 1990) and exposure directly to film. As a control for specificity, the anti-RII antiserum was preincubated with a 50-fold excess of recombinant RII subunit. Assays for AKAP95 followed similar procedures, using a rabbit antibody directed against this protein (Coghlan *et al.*, 1994; 1/500 dilution) as the primary antibody.

RII overlay procedure. Solid phase binding overlays were performed by the method of Lohmann *et al.* (1984) with modifications described by Bregman *et al.* (1989). Briefly, protein samples from nuclear and extranuclear fractions from an equivalent of 3×10^5 cells of each type were separated by 7.5% SDS–PAGE (acrylamide/bisacrylamide, 73/1) and transferred to nitrocellulose. The immobilized protein was partially renatured by incubation in a blocking solution of powdered milk before reaction with ^{32}P-labeled RII probe. After extensive washing to remove uncomplexed RII, AKAPs were detected by autoradiography (Carr and Scott, 1992). For competition experiments, [^{32}P]RII was preincubated with a 14-residue sequence representing the conserved RII binding domain of the AKAP designated Ht31, before using it in the overlay procedure (Carr *et al.*, 1992a).

Nuclear matrix preparation. Precartilage nuclei collected from stage 25 chicken wing tips were extracted as described (Wolda and Glomset, 1988; Nakayasu and Berezney, 1991). Briefly, the nuclei were resuspended in buffer A containing 200 μg/ml DNase (Sigma), 200 μg/ml RNase (USB), and 1 mM PMSF on ice for 1 hr, followed by centrifugation in the Sorvall SS-34 rotor for 5 min at 5000 rpm. The pellet was then treated with 0.2% Triton X-100 (Sigma) for 15 min, followed by centrifugation for 10 min at 6000 rpm, and finally incubated in 1.6 M NaCl for 30 min, followed by centrifugation for 15 min at 6000 rpm. The nuclease-soluble, Triton-soluble, and salt-soluble fractions were collected as supernatants from the successive cycles of centrifugation. The final pellet represented the nuclear matrix. Fractions were separated by SDS–PAGE and transferred to nitrocellulose. AKAPs were detected by the [^{32}P]RII overlay procedure.

Primary cell culture. Primary cultures were prepared by isolating distal tips from stage 25 chick wing buds and dissociating mesenchymal cells with trypsin–EDTA as described (Frenz *et al.*, 1989a,b; Downie and Newman, 1994). Cells suspended at 2.5×10^7 cells per ml were deposited as 10-μl droplets in 24-well tissue culture plates (Costar). After cell attachment (1 hr), each well received 1 ml of serum-free medium (60% Ham's F12 (Gibco)/40% Dulbecco's modified minimal essential medium (Sigma), 5 μg/ml insulin, 100 nM hydrocortisone, 50 μg/ml L-ascorbic acid, 5 μg/ml chicken transferrin (Sigma) (Paulsen *et al.*, 1988)). Plates were incubated at 37°C, 5% CO_2, with daily changes of medium. At Days 1, 3, and 6 after plating, cells were harvested by scraping into NPB, and nuclei were prepared as described above.

RESULTS

Changes in Intracellular Compartmentalization of Protein Kinase A during Cartilage Differentiation

Nuclear and extranuclear fractions of precartilage and cartilage tissues (Fig. 1) were examined for the presence of substrates and components of PKA. Because the integrity of the cell fractionation process was critical for these studies, we used a number of markers to confirm the authenticity of the fractions. Lactate dehydrogenase activity (a cytosolic marker) was undetectable in the precartilage nuclear fraction, compared with 90.4 units per microgram of total protein in the extranuclear fraction. In cartilage, LDH activity in the extranuclear fraction was 12-fold greater that of the nuclear fraction on a per protein basis. The precartilage

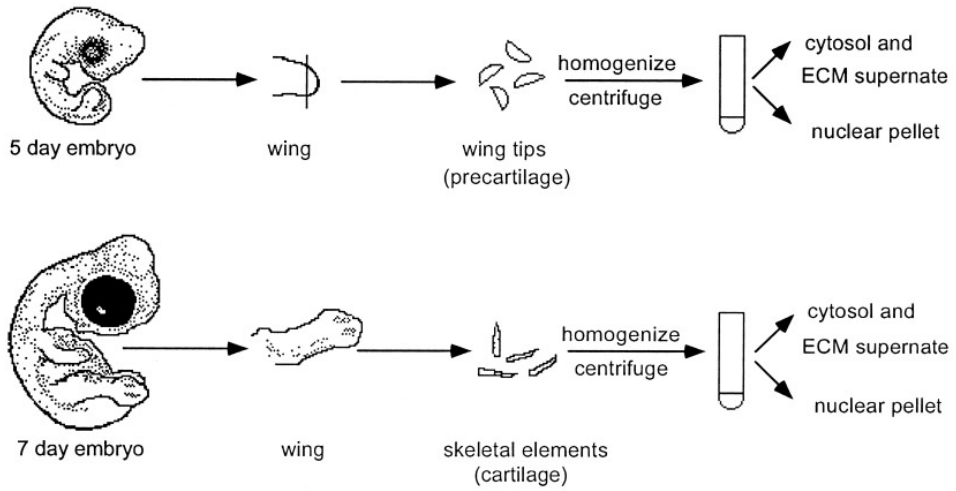

FIG. 1. Schematic representation of embryonic sources and isolation procedures for the precartilage and cartilage tissues analyzed in this study.

nuclear fraction was therefore free of cytosolic components, and the cartilage nuclear fraction essentially so. In addition, CREB protein (mainly present in precartilage in this lineage) was at least 10-fold more concentrated in the precartilage nuclear fraction relative to the extranuclear fraction, and fos protein (present in cartilage, but not precartilage tissue) was at least 50-fold more concentrated in the nuclear relative to the extranuclear fraction, as determined by immunoblotting (not shown). Electron microscopy confirmed that there was no endoplasmic reticulum associated with the nuclei of either cell type (not shown).

As reported previously, an ~35-kDa protein (p35) was phosphorylated in a cAMP-dependent fashion in isolated precartilage nuclei (Leonard and Newman, 1987). This phosphorylation was inhibited by PKI(11-24), an inhibitor of PKA (Walsh *et al.*, 1971; Scott *et al.*, 1986) (not shown). Precartilage nuclear and extranuclear fractions had different patterns of *in vitro* phosphorylated proteins (Fig. 2A). The extent of phosphorylation of p35 was markedly increased in the presence of cAMP in both nuclear and extranuclear fractions isolated from precartilage tissue (Fig. 2A, lanes 2 and 4). In cartilage, p35 was phosphorylated in the presence of cAMP in the extranuclear fraction (Fig. 2B, lane 4) but not in the nuclear fraction (lane 2).

Because the phosphorylated form of p35 was previously found to coelectrophorese with an abundant, broadly focused, basic protein by equilibrium two-dimensional gel electrophoresis (Leonard and Newman, 1987), we analyzed phosphoproteins from the precartilage and cartilage nuclei using NEPHGE, which provides better resolution of basic proteins (O'Farrell *et al.*, 1977) (Fig. 3). A discrete spot (p35, arrowhead) was confirmed to be phosphorylated in a cAMP-dependent fashion in precartilage nuclei (Fig. 3, top) but not in cartilage nuclei (Fig. 3, bottom). The cAMP-dependently phosphorylated p35 identified in precartilage nuclei on both one- and two-dimensional gels comigrated when excised from the respective gels and reelectrophoresed on a second one-dimensional gel (not shown).

It was unclear whether failure of p35 to become phosphorylated in cartilage nuclei in response to cAMP was due to its absence there or to the lack of the requisite enzyme. We therefore performed the phosphorylation assay with cartilage nuclei in the presence of exogenous PKA catalytic subunit. Under these conditions p35 was phosphorylated in cartilage nuclei (Fig. 4A). The phospho-p35 spots isolated from precartilage and cartilage nuclei had identical tryptic maps (not shown). Therefore, lack of phosphorylation of p35 in normal cartilage nuclei was due to the absence of PKA and not due to occupancy of the phosphorylation sites by phosphate. Although it remained possible that PKA was present, but inactived, in cartilage nuclei, immunoblotting with an antibody against this enzyme's catalytic subunit detected a protein of the expected molecular weight of ~41 kDa (Edelman *et al.*, 1987) in precartilage nuclei, but not in cartilage nuclei (Fig. 4B).

Distribution of Regulatory Subunits of PKA in Limb Precartilage and Cartilage Cells

A photoaffinity probe, [^{32}P]8-azido-cAMP, was used to detect the presence of regulatory subunits of cAMP-dependent protein kinase in nuclear and extranuclear fractions of precartilage (Fig. 5A; lanes 1–4) and cartilage (lanes 5–8). Excess nonradioactive cAMP was used to block specific binding of the photoaffinity probe to its binding proteins (lanes 2, 4, 6, and 8). In the precartilage nuclear fraction (lane 1), several proteins bound [^{32}P]8-azido-cAMP in a manner that was blocked by adding excess nonradioactive cAMP. Affin-

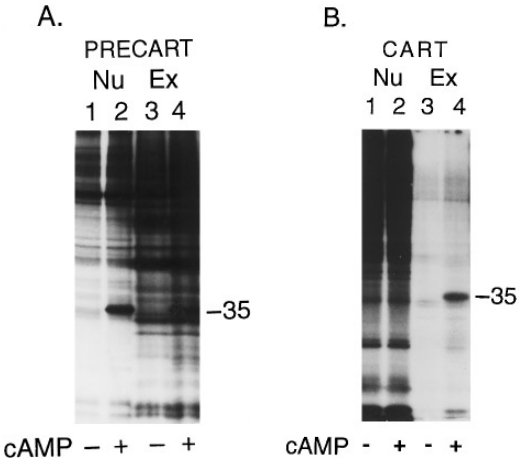

FIG. 2. cAMP-dependent protein phosphorylation in nuclear and extranuclear fractions of precartilage and cartilage cells. Nuclear (Nu) and extranuclear (Ex) proteins (equivalent of 1.2×10^6 cells/lane) were subjected to phosphorylation for 10 min in the absence (lanes 1, 3) and presence (lanes 2, 4) of 10 μM cAMP. Each sample contained 5 μM [^{32}P]ATP. Proteins were separated by electrophoresis in a 12.5% SDS–PAGE. Phosphorylation was evaluated by autoradiography. (A) Precartilage nuclear and extranuclear fractions. p35 is phosphorylated only in the presence of cAMP in both nuclear (lane 2) and extranuclear (lane 4) fractions. This result is typical of three experiments. (B) Cartilage nuclear and extranuclear fractions. In cartilage, p35 was phosphorylated in the extranuclear fraction in the presence of cAMP (lane 4), but not in nuclei under the same conditions (lane 2). This result is typical of two experiments.

ity-labeled proteins of ~53–54 kDa that were intensely and specifically labeled migrated similarly to recombinant RII (lane 1). A greatly decreased amount of this labeled product was observed in the extranuclear fraction of precartilage cells (lane 3). The relative distribution of labeled 53- to 54-kDa bands was reversed in the cartilage fractions: these proteins were present in very reduced amounts in the cartilage nuclear fraction (lane 5), but in high levels in the extranuclear fraction (lane 7). An affinity labeled protein of M_r ~49 kDa, the labeling of which was completely blocked by nonradioactive cAMP in the extranuclear fractions of both cell types (lanes 3, 4; 7, 8), may have been RI, although breakdown products of RII have also been reported in this molecular weight range (Beebe and Corbin, 1986). A doublet at this position was partially competed by nonradioactive cAMP in precartilage nuclei (lanes 1 and 2), but not in cartilage nuclei (lanes 5 and 6).

To confirm the presence of RII isoforms in precartilage and cartilage cells, RII antiserum was used for immunodetection of proteins from the corresponding tissue fractions (Fig. 5B). The affinity labeled 53- to 54-kDa protein in various precartilage and cartilage fractions was recognized by the anti-RII antibody. RII was abundant in the precartilage nuclear fraction and very scarce in the cartilage nuclear fraction. Both the precartilage and cartilage extranuclear fractions contained RII, although it was more abundant in the latter than the former. This difference in relative distribution was in agreement with the affinity labeling data shown above (Fig. 5A). These results were confirmed with three additional anti-RII antibodies. Although all of these antibodies detected additional bands of higher and lower M_r (not shown), all immunoreactivity to the 53- to 54-kDa bands in our samples was blocked by preincubation of the antibodies with recombinant bovine RII. Electron microscopic immunolocalization studies making use of these antisera (not shown) indicated that the nuclear immunoreactivity in precartilage cells was localized within the nucleus and not to the perinuclear regions.

We considered the possibility that RII might have adventitiously redistributed to the nuclei during the homogenization of the tissue in aqueous buffer. This was of particular concern when we found that precartilage nuclei contained significant amounts of a protein with binding affinity for RII (see below). We therefore also prepared precartilage nu-

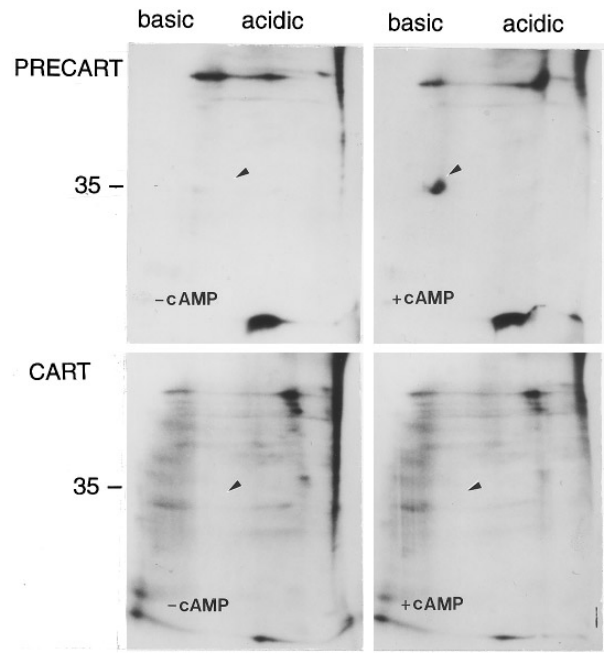

FIG. 3. Two-dimensional nonequilibrium pH gradient gel electrophoretic (NEPHGE) analysis of cAMP-dependent protein phosphorylation in precartilage (top) and cartilage (bottom) nuclei. Nuclei isolated from 2.0×10^6 precartilage cells/gel and 2.2×10^6 cartilage cells/gel were incubated with [^{32}P]ATP for 10 min without or with 10 μM cAMP, followed by 30 μg/ml DNase I treatment at 37°C for 30 min as described under Materials and Methods. Nuclear proteins of each cell type were analyzed by NEPHGE followed by autoradiography. p35 was phosphorylated in a cAMP-dependent fashion in precartilage nuclei, but not in cartilage nuclei. This result is typical of two experiments.

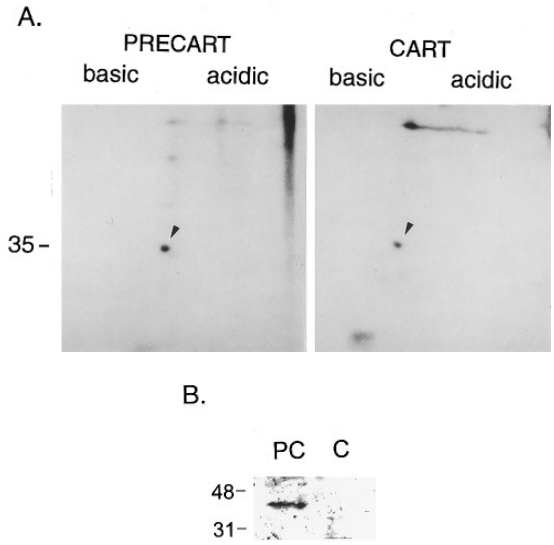

FIG. 4. (A) Phosphorylation of precartilage and cartilage nuclear proteins in the presence of catalytic subunit of PKA. Proteins from 5.7×10^6 precartilage nuclei (left) and 4.0×10^6 cartilage nuclei (right) were separated by NEPHGE and analyzed by autoradiography. p35 was phosphorylated in both nuclear preparations. (B) Immunoblot analysis of precartilage (PC) and cartilage (C) nuclei with an antibody directed against the catalytic subunit of PKA. An immunoreactive ~41-kDa protein was present in precartilage, but not cartilage, nuclei. Proteins from approximately 2×10^6 nuclei from each cell type were separated on a 12.5% SDS–polyacrylamide gel and transferred to nitrocellulose. Detection was by ECL.

clei by the nonaqueous procedure of Lynch *et al.* (1975) and looked for RII in the nuclear and extranuclear fractions. The distribution of RII in these samples (Fig. 5B, last two lanes) was virtually identical to the that in the standard preparations (Fig. 5B, first two lanes), indicating that RII did not redistribute during the standard nuclear isolation procedure.

Distribution and Localization of AKAPs in Limb Precartilage and Cartilage Cells

AKAPs were detected by overlaying protein blots with radiolabeled RII (Fig. 6A). An AKAP of ~150 kDa was found in nuclear samples, but not in extranuclear samples. Like RII, this protein, designated nAKAP150, was abundantly labeled in precartilage nuclei, but present in very reduced amounts in cartilage nuclei. In competition experiments nAKAP150 labeling was completely blocked by preincubation of [^{32}P]RII with a peptide comprising the RII binding region of a previously described AKAP, designated Ht31 (Carr *et al.*, 1991; 1992a) (Fig. 6B). Lack of nAKAP in cartilage nuclei did not reflect its loss during preparation, since it was not detected in cartilage tissue directly solubilized with SDS gel sample buffer (not shown). Moreover, the presence of nAKAP150 in precartilage nuclei was not a consequence of redistribution during the fractionation procedure. This was demonstrated by further fractionation of precartilage nuclei into nuclease soluble, Triton soluble, salt soluble, and nuclear matrix fractions. nAKAP150 was highly enriched in the nuclear matrix fraction in which 1% or less of nuclear proteins remained after treatment with DNase, RNase, Triton, and high salt. This protein was present in very reduced amounts in the salt soluble and Triton soluble fractions and was undetectable in the nuclease soluble fraction (Fig. 7).

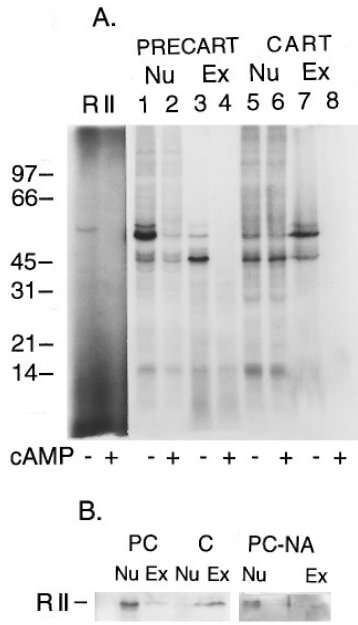

FIG. 5. (A) Photoaffinity labeling of cAMP binding proteins in nuclear (Nu) and extranuclear (Ex) fractions of precartilage and cartilage cells. Proteins from precartilage nuclear (lanes 1, 2) and extranuclear (lanes 3, 4) fractions, and cartilage nuclear (lane 5, 6) and extranuclear fractions (lanes 7, 8) were incubated with [^{32}P]8-azido-cAMP as described under Materials and Methods. Excess nonradioactive cAMP (100 μM) was used to block specific photoaffinity labeling of cAMP to its binding proteins (lanes 2, 4, 6, and 8). Samples were prepared from approximately 1.5×10^6 cells of each cell type. After photoaffinity labeling proteins were separated by electrophoresis on a 12.5% SDS–polyacrylamide gel and analyzed by autoradiography. Recombinant bovine RII was used as a positive control. (B) Immunoblot analysis of RII in nuclear and extranuclear fractions of precartilage and cartilage cells. Precartilage (PC) nuclear and extranuclear proteins and cartilage (C) nuclear and extranuclear proteins (equivalent of 2×10^6 cells/lane) were separated by electrophoresis on a 12.5% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. Detection was with an ^{125}I-labeled secondary antibody followed by autoradiography. The last two lanes contain nuclear and extranuclear fractions, respectively, of precartilage mesenchyme fractionated by a nonaqueous procedure (PC-NA) (see Materials and Methods). Electrophoresis, transfer, and primary antibody were as above, but detection was by ECL.

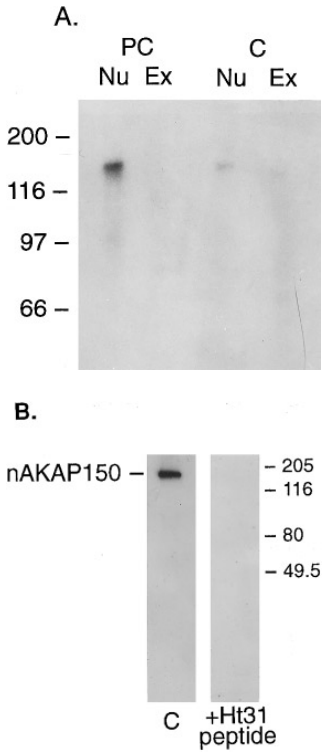

FIG. 6. (A) Distribution of AKAPs in nuclear (Nu) and extranuclear (Ex) fractions of precartilage (PC) and cartilage (C) cells. Samples from an equivalent of 3×10^5 cells/lane of each type were separated by electrophoresis on a 7.5% SDS–PAGE. After electrotransfer to nitrocellulose AKAPs were detected as described under Materials and Methods. This result is typical of three experiments. (B) Binding of [^{32}P]RII to AKAPs in precartilage nuclear sample (left, control) was blocked by preincubation of [^{32}P]RII with Ht31 peptide (right).

Changes in Nuclear RII and AKAPs during Chondrogenesis in Vitro

Mesenchymal cells isolated from 5-day wing buds (stage 24–25; Fig. 1) undergo chondrogenesis when cultured at high density in serum-free medium (Downie and Newman, 1994). By Day 6 in culture the tissue has entirely differentiated into cartilage. Immunoblot analysis of RII subunit was performed on nuclei isolated from wing tip mesenchyme after 1, 3, and 6 days of culture. Nuclear RII was most abundant at Day 1 and progressively decreased during chondrogenesis (Fig. 8A). Assays for AKAPs in nuclear and extranuclear fractions prepared from 3- and 6-day cultures showed that nAKAP150 decreased dramatically from Day 3 to Day 6 (Fig. 8B). There were no AKAPs detected in the extranuclear fractions at any of the stages assayed.

DISCUSSION

Limb bud precartilage cells undergo a transient condensation process that appears to be required for chondrogenesis *in vivo* (Hall and Miyake, 1992; Newman and Tomasek, 1996) and unless circumvented experimentally (Zanetti and Solursh, 1984), *in vitro*. This process may reflect, and provide the basis for, cell–cell and/or cell–matrix interactions (Frenz *et al.*, 1989a,b; Leonard *et al.*, 1991; Widelitz *et al.*, 1993; Oberlender and Tuan, 1994; Downie and Newman, 1994), cytoskeletal-linked changes in cell shape (Zanetti and Solursh, 1984), and prostaglandin release (Biddulph *et al.*, 1988). The elevation of intracellular cAMP levels, which accompanies condensation and may be a consequence of this process, has been implicated in promoting chondrogenesis by the stimulation of cartilage-specific gene expression (Kosher *et al.*, 1986a,b). The phosphorylation of p35 is the only nuclear event in precartilage cells that has so far been tied directly to the elevation of cAMP (Leonard and Newman, 1987; this study).

Cyclic AMP regulates a myriad of cellular processes through the cAMP-dependent protein kinase (Lee, 1991). In many cases, activation of PKA leads to altered patterns of gene transcription by phosphorylation of nuclear proteins (Brindle and Montminy, 1992). On the basis of spatially resolved microinjection studies using fluorescently labeled PKA catalytic and type I regulatory subunits, and differentiated fibroblasts or neuronal cells, it has been suggested that the regulatory subunits and the intact holoenzyme are unable to enter the nucleus, whereas the free catalytic subunit can traverse the nuclear pores when dissociated from its cytoplasmic anchors (Meinkoth *et al.*, 1990; Bacskai *et al.*, 1993; Harootunian *et al.*, 1993). However, other studies have detected RII isoforms in cell nuclei (Tortora and Cho-Chung, 1990), and they may be required for particular transcriptional responses (Lee, 1991). Our finding that RII is highly enriched in precartilage nuclei, but essentially absent

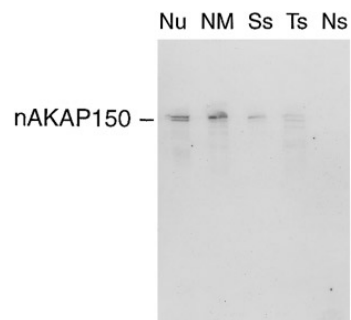

FIG. 7. Distribution of nAKAP150 in precartilage nuclear subfractions. Precartilage nuclei were treated with nucleases, Triton X-100, and high salt. Nuclease-soluble fraction, Triton-soluble fraction, salt soluble fraction, and Triton/salt-insoluble fraction (nuclear matrix) were collected by centrifugation. Fractions were separated by SDS–PAGE and transferred to nitrocellulose. AKAPs were detected by [^{32}P]RII overlay procedure. Nu, intact nuclei; NM, nuclear matrix fraction; Ss, salt soluble fraction; Ts, Triton soluble fraction; Ns, nuclease-soluble fraction.

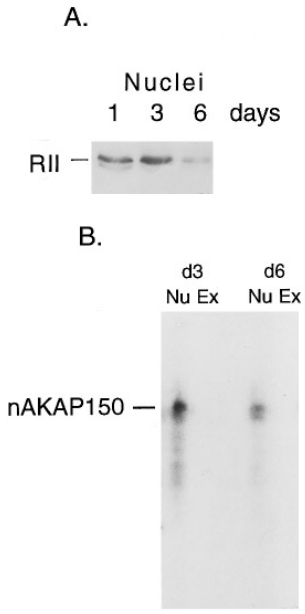

FIG. 8. Distribution of RII and nAKAP150 during chondrogenesis *in vitro*. Cells were cultured in serum-free medium for 1, 3, and 6 days. Nuclear and/or extranuclear fractions from each day were separated by electrophoresis and transferred to nitrocellulose membranes. (A) Immunoblot analysis of RII in Days 1, 3, and 6 nuclei. Detection was by ECL. (B) Analysis of AKAPs in Days 3 and 6 nuclear (Nu) and extranuclear (Ex) fractions. AKAPs were detected by overlaying blot with [^{32}P]RII, followed by autoradiography.

in cartilage nuclei, suggests that nuclear translocation of PKA regulatory subunits may occur transiently during cartilage differentiation. The developmental regulation of AKAP150 could represent a special mechanism to concentrate PKA in the precartilage nucleus during the very stages at which cAMP is eliciting cartilage-specific gene expression (Kosher *et al.*, 1986a,b).

In other cell types the localization of type II PKA to specific cellular compartments appears to depend on the interaction of the RII subunit with specific AKAPs (Lohmann *et al.*, 1984; Sarkar *et al.*, 1984; Scott, 1991). Several such anchoring proteins have been identified and characterized. Cytoskeletal attachment of type II PKA occurs through interactions between RII and microtubule-associated protein 2 (MAP2) (Theurkauf and Vallee, 1982). RII also associates with an A-kinase anchoring protein AKAP75 (formerly designated bovine brain P-75) (Sarkar *et al.*, 1984; Leiser *et al.*, 1986). Several AKAP75 homologs, ranging in size from M_r 60 to 150 kDa have been reported in different species and may represent members of a family of structurally related RII-anchoring proteins (Bregman *et al.*, 1989; Carr *et al.*, 1992a; Hirsch *et al.*, 1992). An AKAP, designated Ht31, isolated by screening a human thyroid cDNA expression library, contains a 14 residue sequence that is similar to RII binding domains of MAP2, AKAP75, and AKAP150 (Carr *et al.*, 1991; 1992a). Competition experiments reported here indicate that nAKAP150 also binds to this same region of RII (Fig. 6B).

A survey of nine different bovine tissues showed that the AKAPs are restricted in their tissue distribution and suggested that type II PKA holoenzyme may be specifically targeted to different locations in each type of cell (Scott *et al.*, 1990). When bovine AKAP75 was overexpressed in a human cell line, stably transfected cells had more than 90% of their RII subunits transferred from a cytosolic to a particulate pool. A corresponding number of non-PKAI-associated catalytic subunits were also transferred to this pool (Ndubuka *et al.*, 1993), indicating that AKAPs represent a means for targeting PKA catalytic activity. This is of particular significance in light of the unexpected finding that PKA can be catalytically active as a holoenzyme (Yang *et al.*, 1995). In the case of precartilage mesenchymal cells this targeting appears to be confined to the nucleus; using the solid phase assay we have been unable to detect any AKAP other than nAKAP150 in precartilage tissue.

Like another recently characterized nuclear AKAP, designated AKAP95 (Coghlan *et al.*, 1994), nAKAP150 is highly enriched in the nuclear matrix (Fig. 7). However, nAKAP150 and AKAP95 do not appear to be related; in preliminary experiments we have found no immunoreactivity to an anti-AKAP95 antibody in precartilage nuclei.

Both RII and nAKAP150 were found in precartilage nuclei, with only trace amounts being present in the nuclei of cartilage cells. Correspondingly, nuclear RII and nAKAP150 both decreased in abundance during chondrogenesis *in vitro*. We suggest that nAKAP150 may function in the spatiotemporal regulation of events in precartilage mesenchymal tissue by targeting regulatable PKA (e.g., holoenzyme) to the precartilage nucleus, thereby enabling it to respond in a rapid and precise manner to transient increases in cAMP. This would be of particular importance in the regulation of skeletal pattern formation, where adjacent blocks of tissue must undergo reliable all-or-none decisions concerning whether or not to enter the chondrogenic pathway (Newman, 1988, 1993).

In a previous study (Leonard and Newman, 1987), p35 was identified with PCP 35.5b, a basic protein that comigrated with it on equilibrium two-dimensional gels of precartilage nuclear proteins. The corresponding stained spot was not detected in gels containing nuclear proteins prepared from a mixture of embryonic limb and vertebral cartilages. The present study employed nonequilibrium gel electrophoresis, which provides superior resolution of basic proteins. Moreover, the cartilage nuclear proteins in the present study were isolated only from embryonic limb cartilage, which represents a more authentic end point for the differentiation of limb precartilage cells. These more definitive studies have shown that the PKA substrate protein p35 is present in limb cartilage nuclei, although it cannot be phosphorylated in response to cAMP there, because of lack of PKA. Since p35 in cartilage nuclei could be phos-

TABLE 1
Summary of Distribution of p35, cAMP-Dependent p35 Phosphorylation, PKA Subunits, and nAKAP150 in Precartilage and Cartilage Cells

	Precartilage		Cartilage	
	Nuclei	Cytosol[a]	Nuclei	Cytosol[a]
p35	+	+	+	+
cAMP-dependent p35 phosphorylation[b]	+	+	−	+
C	+	ND	−	ND
RII	+++	+	+/−	++
nAKAP150	+++	−	+/−	−

Note. C, PKA catalytic subunit; ND, Not determined.
[a] Extranuclear fraction.
[b] Detectable phosphate incorporated by isolated nuclei.

phorylated by the addition of exogenous catalytic subunit of PKA (Fig. 4A), the phosphorylation of this protein during cartilage differentiation appears to be primarily regulated by the nuclear localization of the cAMP-dependent protein kinase during early stages in the development of this lineage. The data presented here, summarized in Table 1, strongly support this hypothesis and suggest a decisive role for the developmentally regulated appearance of nAKAP150 in this process. Because p35 was phosphorylated by exogenous PKA catalytic subunit in precartilage and cartilage nuclei at the same sites (by tryptic mapping), and to similar extents (Fig.4A), it is likely that p35 is dephosphorylated prior to terminal differentiation, and that generation of phospho-p35 during chondrogenesis is therefore a transient event. Whether the phosphorylation of p35 is the primary function of the nuclear import of PKA during chondrogenesis and whether this event mediates the cAMP regulation of cartilage-specific gene expression remain to be determined.

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