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cAMP, an Activator of Protein Kinase A, Suppresses the Expression of Sonic Hedgehog

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In *Drosophila,* it has been shown that protein kinase A and hedgehog have antagonistic actions during the formation of imaginal disks. In vertebrate skin, sonic hedgehog is expressed specifically in the feather bud epithelia. Using an in vitro explant culture model we showed that dibutyryl cAMP, a protein kinase A (PKA) activator, suppresses the expression of Sonic hedgehog, (Shh) and continuous feather growth. The results suggest that Shh and PKA also have antagonistic action during vertebrate skin morphogenesis.

In *Drosophila,* Hedgehog (hh) belongs to the segment polarity class of genes and encodes a secreted protein which plays a role in extracellular signalling pathways (For reviews see 1–3). Specifically, it is involved in the establishment of polarity within embryonic segments (4), development of imaginal disks (5), and the progression of the morphogenetic furrow in the developing eye (6,7). Recently, work from several groups showed that in the anterior imaginal disk, cells which lack cAMP-dependent protein kinase (PKA) express decapentaplegic (dpp) and wingless (wg) (8–10). These cells normally express dpp and wg only in the presence of hh derived from the posterior imaginal disk. Thus the absence of PKA activity could substitute for hh activity. It was concluded that the presence of PKA normally inhibits the expression of dpp and wg, and that hh acts antagonistically to PKA.

One of the vertebrate homologs of *Drosophila* hh is called sonic hedgehog (Shh) and is expressed in the posterior limb bud, notochord, and floor plate (11–14). In these sites of organogenesis, Shh exhibits powerful morphogenetic activity during tissue interaction. Recently it was shown that Shh is also expressed in the epithelia of the developing feather buds (15, 16).

In our laboratory, we have been interested in studying molecules involved in feather morphogenesis (17, 18). We have previously observed that addition of forskolin, an activator of adenylyl cyclase, or dibutyryl cAMP (db-cAMP), an activator of PKA, to stage 34 (E8) skin culture media, results in the stimulation of mesenchymal condensation of feather buds (19). In addition we have found that db-cAMP or forskolin can nearly completely inhibit the expression of homeobox genes Msx1 and Msx2 present in the epithelium of growing feather buds (20).

In vertebrates, the relationship between PKA and Shh has not been established. New evidence suggests that Shh has a role in growth and development of feather (15, 16). Since we have observed that activators of PKA, in addition to stimulating mesenchymal condensation, inhibit feather growth, we wondered if PKA can also inhibit Shh expression. In this report we used an in vitro organ culture model in order to investigate the effect of db-cAMP on the expression of Shh. The results suggest that PKA antagonizes Shh by inhibiting its expression.

MATERIALS AND METHODS

Embryos. White leghorn fertilized chicken eggs were obtained from SPAFAS (Connecticut). Eggs were incubated at 37°C and embryos were staged according to Hamburger and Hamilton (21).

Serum free skin explant cultures. Dorsal skins between the lower neck and tail were removed from eight day old White

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Leghorn chicken embryos in Hank's balanced saline solution (HBSS) and laid down on culture inserts (Falcon). Culture inserts were placed in six well dishes (Falcon), containing 2 ml/well of Dulbecco's modified eagle medium (DMEM) supplemented with 0.1% gentamicin. For activation of adenylyl cyclase or protein kinase A, the media was additionally supplemented with 20 μM forskolin or 1 mM dibutyryl cAMP respectively. The cultures were incubated at 37°C at an atmosphere of 5% CO_2 and 95% air for 5 days. Subsequently the skin explants were prepared for in situ hybridization.

Whole mount in situ. Whole mount in situ hybridization protocols were based on Sasaki and Hogan (22) with minor modifications. Following fixation and bleaching, tissues were hybridized overnight at 70°C with 2 μg/ml of probe. These tissues were subsequently incubated with anti-digoxigenin Fab conjugated to alkaline phosphatase (Boehringer Mannheim). Color was developed by addition of alkaline phosphatase substrate NBT/BCIP (Promega). To prepare the antisense RNA probe, plasmid PHH-2 (a kind gift of Drs. Robert Riddle, Randy Johnson and Cliff Tabin) was linearized with HindIII and transcribed with T3 RNA polymerase in a standard transcription reaction.

PKA assay. PKA assays were performed according to manufacturer's protocols (Gibco BRL) on stages 29, 31, and 34 (E6, E7, and E8) chicken dorsal skin extracts. The synthetic heptapeptide Kemptide, which has a PKA substrate consensus sequence, served as the PKA-specific substrate and a synthetic 17-amino acid peptide (PKI(17aa)),derived from the protein kinase inhibitor, was used as a specific inhibitor of PKA. Incorporation of ^{32}P into substrates indicated the kinase activity of the extracts. Activities obtained in the presence of PKI(17aa) were assumed to be PKA-nonspecific activity and therefore subtracted from the values obtained in the absence of it. The enzyme activity units were obtained by converting the amount of radioactive phosphate transferred to the substrate into PKA enzyme activity units according to the manufacturer's protocol (Gibco BRL). The amount of protein extract used for each determination was 10 μg as determined by Bradford assay (BioRad). Six independent experiments,using extracts derived from three different extractions, were carried out which showed similar trends. We did not pool the data from the independent experiments together because the absolute values varied. However, the relative relationship was similar and therefore we showed one exemplary experiment.

RESULTS

In the present study the effect of PKA activation on the expression of Shh in cultured feather buds was studied. Since, in *Drosophila*, it was found that PKA is antagonistic to hh (8–10), we wondered if in vertebrates, activation of PKA is capable of inhibiting the expression of Shh.

When Dibutyryl cAMP (db-cAMP) is added to the media stage 34 (E8) dorsal chicken skin explant, round large symmetric buds are formed (19). This, and other evidence, indicate that db-cAMP stimulates the mesenchymal condensation of feather buds (19). In addition, we have observed that db-cAMP or forskolin inhibit the growth of feather buds.

dbcAMP (1 mM) was added to culture media of stage 34 (E8) dorsal chicken skin (Fig. 1A) and the skin was grown for 5 days at 37°C. In the presence of db-cAMP, large symmetric round buds with stunted growth were formed (Fig. 1B, 1C). No further feather bud growth was seen even after 14 days of incubation, although no more dbcAMP was added to the culture media after it was applied at the first day of incubation. Forskolin, an activator of adenylyl cyclase, had a very similar effect (19).

We tested the expression of Shh in skins using the whole mount in situ hybridization assay using digoxigenin-labeled probe. We found Shh expression in the distal feather buds epithelia in the control skins (Fig. 2A, 2B). In the db-cAMP-treated skin, the expression of Shh was suppressed (Figure 2C, 2D). The suppression was more pronounced at the posterior of the dorsal skin than the anterior since the posterior is at a younger embryonic age (17).

The suppression of Shh by db-cAMP suggests that PKA has a physiologic role in the control of Shh expression. We have previously shown that stage 31 (E7) chicken dorsal skin has an endogenous PKA activity and that enhancing PKA activity induces dermal condensations (19). In order to find out if there is a developmental regulation of PKA activity, PKA assays were performed on stages 29, 31, and 34 (E6,E7, and E8) chicken dorsal skin extracts. PKA activity appears to decrease progressively from stage 29 to 34 (Figure 3). Interestingly, Shh expression progressively increases during these stages (16). This indicates that there may be a regulatory relationship between PKA and Shh during development.

DISCUSSION

We have earlier shown that addition of db-cAMP or forskolin to cultured embryonic chicken skin lead to the formation of buds that are large, round and symmetrical (19). In the present study

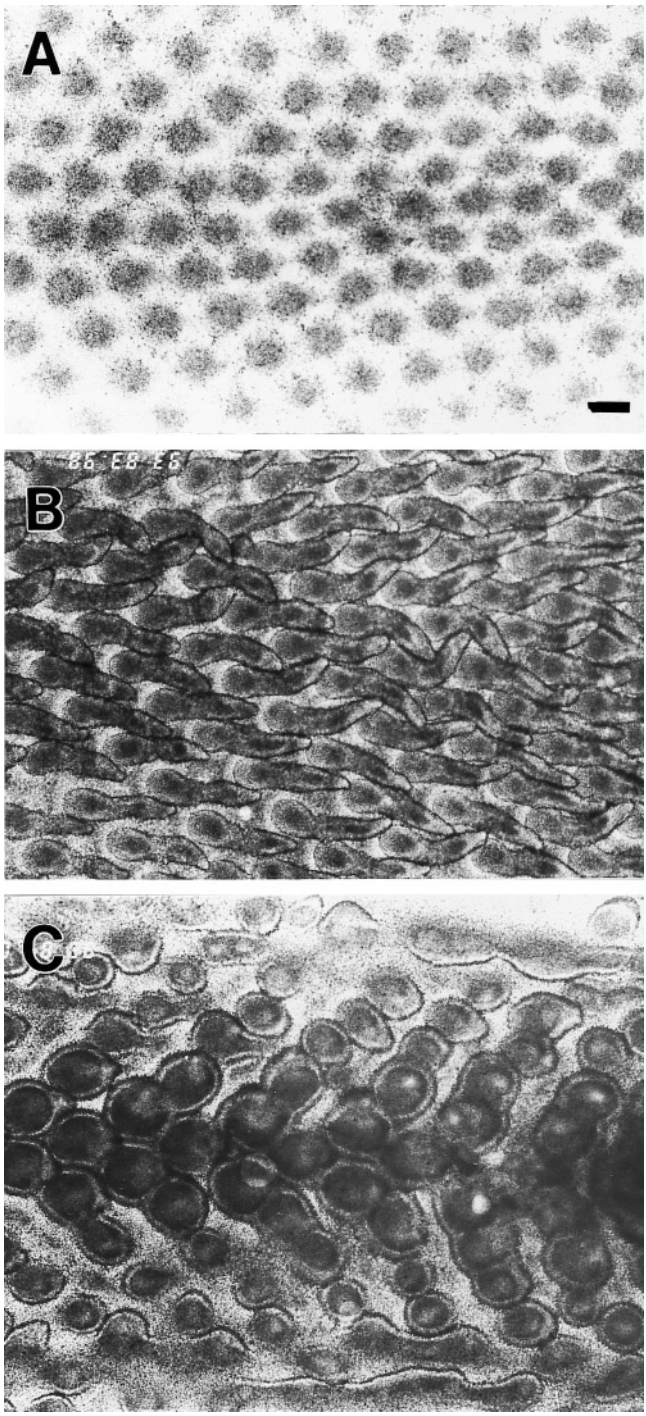

FIG. 1. The effect of the protein kinase A activator, db-cAMP, on feather bud condensation. Whole mount view of the skin explant. Anterior is to the left of the panel. (A) Normal stage 34 (E8) chicken dorsal skin. Formation of dermal condensations can be observed. (B) Normal stage 34 (E8) dorsal skin showing feather bud growth after 5 days of incubation in DMEM at 37°C. (C) db-cAMP-treated stage 34 (E8) skin after 5 days of incubation in DMEM at 37°C. db-cAMP (1 μM) causes the formation of round, symmetric, and nonelongating feather buds. Because feather buds initiate from the midline and spread laterally, a stage 34 (E8) skin exhibits different stages of feather development. Feather buds farthest from the central longitudinal axis are youngest and are affected more dramatically, forming a continuous condensed zone. Size bar, 250 μm.

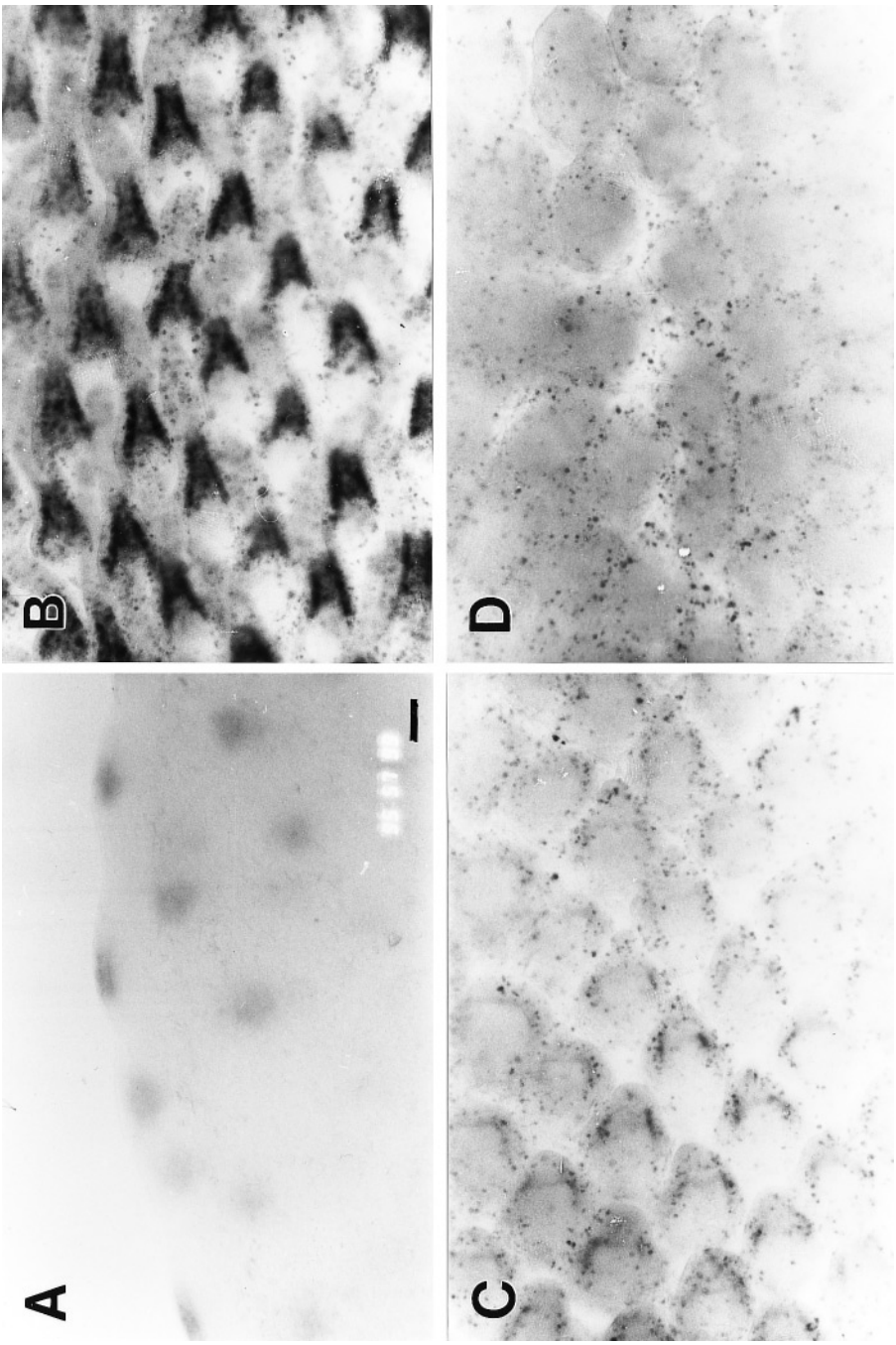

FIG. 2. Whole mount in situ hybridization of skin explants using digoxigenin-labeled antisense Shh probe. (A) Normal expression of Shh in stage 31 (E7) skin in epithelia of the forming feather buds. (B) Expression of Shh in stage 34 (E8) skin after the skin was cultured for 5 days at 37°C. The dark solid areas are where the probe hybridization occurred. Note the expression of Shh in the distal epithelia of feather buds. The black round spots are pigmentation and not where probe hybridized. (C) db-cAMP-treated stage 34 (E8) skin, anterior skin section, cultured for 5 days at 37°C. dbcAMP was used at a concentration of 1 mM. Partial suppression of Shh could be observed. Note that the anterior buds still partially retain their conical shape. There is a good degree of correlation between the change in feather shape and the degree of Shh suppression. (D) db-cAMP-treated stage 45 (E8) skin, posterior skin section, cultured for 5 days at 37°C. The expression of Shh is completely suppressed in the symmetric round buds. Size bar 250 μm.

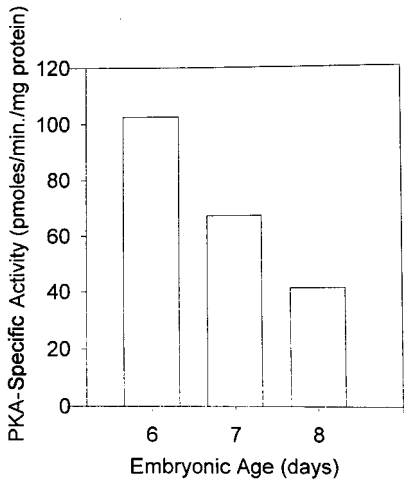

FIG. 3. PKA activity of stages 29, 31, and 34 (E6, E7, and E8) chicken dorsal skin extracts. To measure PKA activity, the PKA specific substrate (Gibco BRL) was incubated with skin extracts and radioactive gamma-labeled ATP. The amount of radioactive phosphate transferred to this substrate was converted to enzyme activity units (see Materials and Methods). A graded reduction of PKA activity at the time when feather buds elongate and anterior–posterior asymmetry is established is observed. This graph is a representative of 6 different determinations using extracts derived from three different extractions. All the experiments showed the same trend. The results were not pooled together because the absolute values varied. For this reason we do not show an error bar.

we also observed that although dbcAMP stimulates mesencymal condensations, it completely inhibits the growth of feather buds. This stunted growth may be due to reduced cell proliferation (20). Thus at the mesenchymal condensation stage, cAMP has a stimulatory effect, and at the elongation phase it has an inhibitory effect. Another way of looking at this is that cAMP causes a blockage between the short feather bud and the long feather bud stages (18).

Since cAMP activates PKA and the activated PKA in turn phosphorylates the cAMP response element binding protein (CREB), we studied the expression of both CREB and phosphorylated CREB (P-CREB) in the growing feather buds (19). We showed that while CREB is ubiquitously expressed, P-CREB is specifically and transiently expressed in the mesenchyme of feather buds, suggesting that enhancement of PKA activity favors the formation of dermal condensation. Furthermore, the expression of P-CREB is lost in the long feather buds, suggesting that the loss of PKA activity is required for cell proliferation and continuous growth (19).

In Drosophila, Li et al. (10) have shown that overexpression of the catalytic subunit of PKA can antagonize hh by inhibiting the expression of patched (ptc). In this report we observed that in chicken feather buds, the expression of Shh transcript is suppressed by db-cAMP and forskolin. Since db-cAMP activates PKA and we showed that there is endogenous PKA activity in the feather bud (19), it is likely that there is an interaction loop between the PKA and Shh pathways during the development of feather buds. This interaction is further supported by our present observation that during embryonic stages 29–34, the activity of PKA is decreased. Because the expression of Shh is increased at the same time (16), PKA may be negatively regulating the expression of Shh.

The data here established that agents that activate PKA in vivo suppress Shh, while concomitantly suppressing further elongation and anterior-posterior development of feather buds. Whether this suppression is direct or indirect will be determined in future experiments.

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