Original Article

Hydrocortisone modulates the expression of c-ets-1 and 72 kDa type IV collagenase in chicken dermis during early feather morphogenesis

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ABSTRACT At the onset of chicken feather morphogenesis, dermal cells migrate along bundles of collagen fibers to colonize areas where bud outgrowth takes place. Chicken embryos treated with hydrocortisone during the critical phase of dermal rearrangement show featherless skin areas in which the dermis exhibits an increase of interstitial collagen. We had previously demonstrated that c-*ets-1* is a nuclear transcription factor expressed in the dermis at the beginning of feather morphogenesis. Here we study, by *in situ* mRNA hybridization, the expression of c-*ets-1* in the dermis of chicken embryos treated with hydrocortisone. We found that, among the two distinct products (p54 and p68) encoded by the chicken c-*ets-1*, the expression of the p68 product increased while expression of p54 decreased after hydrocortisone treatment. Since Ets-1 regulates matrix-metalloproteinases genes, we analyzed the expression of the 72 kDa type IV collagenase in both normal and hydrocortisone-treated embryos. We demonstrated that 72 kDa type IV collagenase mRNA expression decreased in the dermis after hydrocortisone treatment and that its expression correlated with that of p54$^{c\text{-}ets\text{-}1}$. Taken together, these results indicate that hydrocortisone modulates c-*ets-1* expression. In addition, they raise the interesting possibility that c-*ets-1* might be involved in an altered pattern of feather development mediated by the accumulation of collagen due to a decrease in collagenase activities.

KEY WORDS: *dermis, feather morphogenesis, c-ets-1, matrix-metalloproteinase, c-jun , hydrocortisone*

Introduction

In chicken, feather morphogenesis results from interactions between epidermis and dermis. These interactions lead to specific spatio-temporal patterns of cellular division, migration and differentiation. After a first wave of mesenchymal accumulation underneath the epidermis, the formation of epidermal placodes triggers a spatial rearrangement of dermal cells leading to the aggregation of these cells under each epidermal placode (Sengel, 1957, 1970, 1990; Sengel and Rusaouën, 1968; Wessels, 1965; Chuong and Edelman, 1985; Desbiens *et al.*, 1991). At least two distinct extracellular matrix components are involved during this process: fibronectin, which allows cellular adhesion, and interstitial collagen (Mauger *et al.*, 1982), which is arranged in a fibrillar network linking the centers of rising feather rudiments. It has been proposed that dermal cells migrate along these bundles to colonize the sites of aggregation beneath epidermal placodes (Dhouailly, 1984). It has also been reported that hydrocortisone, a glucocorticoid hormone synthesized by adrenal glands, is able to modify feather formation when administered at the onset of dermal cell migration (Züst, 1971). Indeed, treatment with hydrocortisone leads to a depletion of fibronectin and a uniform accumulation of interstitial collagen in the dermal extracellular matrix causing disruption of the collagen fiber network and the subsequent loss of dermal cell migration towards aggregating centers (Demarchez *et al.*, 1984). In addition, the normal proliferation pattern was conspicuously modified in the two skin compartments (Desbiens *et al.*, 1992).

Proto-oncogenes control both cell proliferation and differentiation. During chicken skin development, the expression pattern of three proto-oncogenes encoding nuclear transcription factors exhibit peculiar features: c-*myc* and c-*myb* are expressed in epidermal and dermal cells when a high rate of cellular proliferation is detected. In contrast c-*ets-1* is solely detected in undifferentiated proliferating dermis which undergoes spatial reorganization, and later in endothelial cells which invade newly established feather buds (Desbiens *et al.*, 1991). Since c-*ets-1* is expressed in chicken dermis at the glucocorticoid-sensitive onset of feather morphogenesis (Desbiens *et al.*, 1991; Quéva *et al.*, 1993), we

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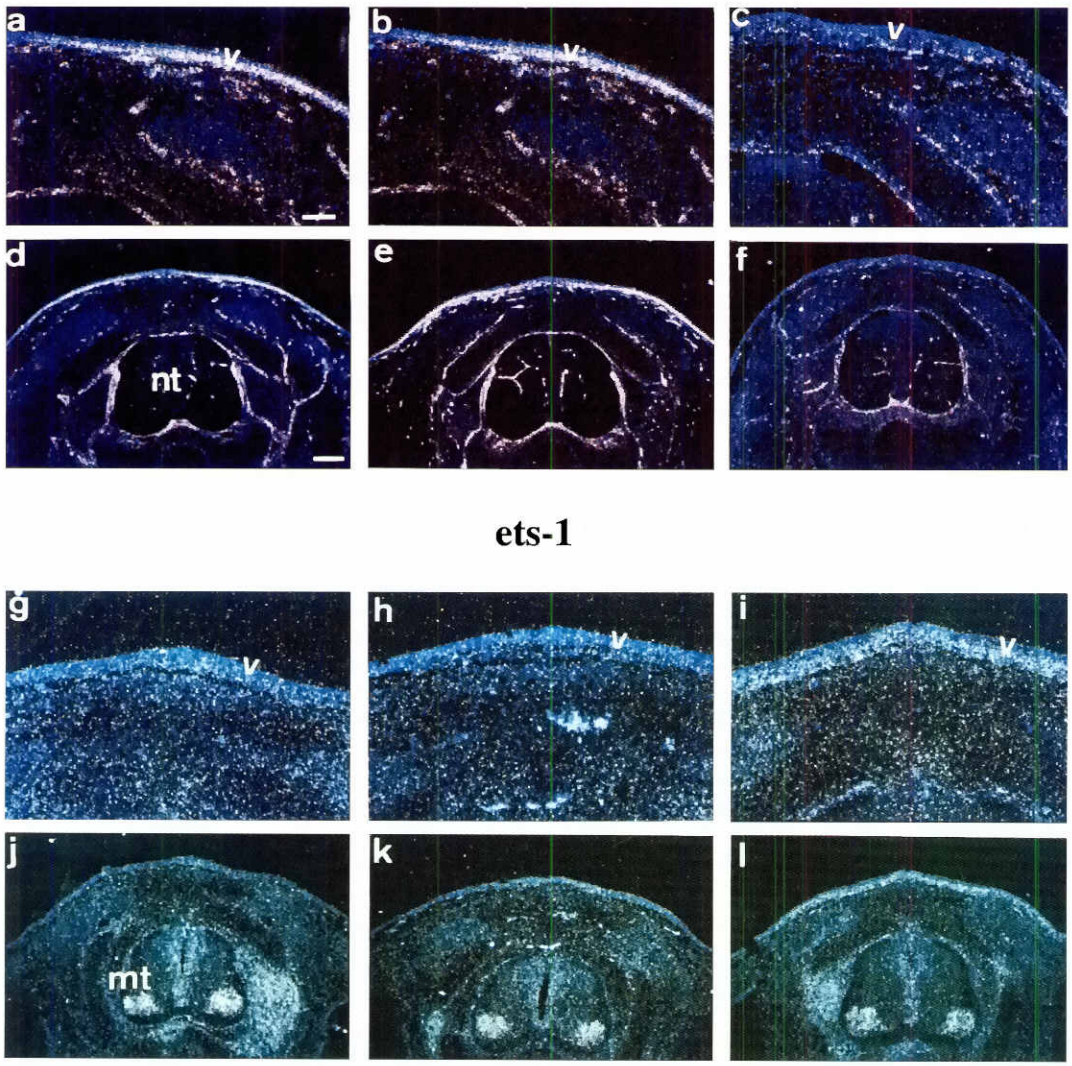

Fig. 1. Expression of Ets1 and Jun in dermis of transverse sections of chicken embryos. *Chicken embryos were treated with PBS as control, or with 0.1 mg of hydrocortisone at E6.5 and fixed 8 h or 24 h (not shown) after injection. Sections from dorsal dermis were hybridized with a sense (not shown) and antisense mRNA common probes for both c-ets-1 transcripts encoding* $p68^{c\text{-}ets\text{-}1}$ *and* $p54^{c\text{-}ets\text{-}1}$ *and for c-jun. As previously described (Desbiens et al., 1991), c-ets-1 transcripts are abundant in the dermis at E6.5* **(a and d)** *and even in the following 8 h* **(b and e)**. *The c-ets-1 signal strongly decreases in the dermis of hydrocortisone-treated embryos (arrowhead)* **(c and f)**. *C-jun is weakly expressed in chicken dermis at E6.5* **(g and j)** *or at E6.5 +8 h* **(h and k)**. *C-jun expression is higher in motoneurons (mt). In hydrocortisone-treated embryos, c-jun is more expressed in the neural tube and in the dermis (compare h and i or k and l). nt, neural tube; mt, motoneurons. Note that panels a,b,c,g,h and i represent an enlargement of the dermis. Bars: a,b,c,g,h,i, 75 μm; d,e,f,j,k,l, 190 μm.*

decided to study the effect of glucocorticoids on c-*ets-1* expression. Ets-1 belongs to a growing family of transcription factors which share the ETS domain, a 85 amino acid strongly conserved in the DNA-binding domain (MacLeod *et al.*, 1992). C-*ets-1* encodes, by alternate promoter usage, two nuclear proteins, p68 and p54 (Crepieux *et al.*, 1993). The two transcription factors contain the same DNA-binding but differ in their transactivation domains: the p68 contains two transactivation domains, one of which is common to the p54 isoform (Schneikert *et al.*, 1992). The p54 and p68 proteins are expressed in a wide population of mesodermal cells of the chicken embryo, particularly in dermis (Quéva *et al.*, 1993).

The Ets-family of transcription factors regulate cellular genes such as *collagenase1* and *stromelysin1* (Gutman and Wasylyk, 1990; Butticè and Kurkinen 1993; Butticè *et al.*, 1996). The two genes belong to a family of matrix-metalloproteases (MMPs) that degrade several components of the extracellular matrix (ECM) such as collagen, fibronectin, laminin, tenascin and proteoglycans. Active turnover of the extracellular matrix molecules occurs in several steps of growth, development and morphogenesis, suggesting that the MMPs play a major role in these processes (Alexander and Werb, 1989; Birkedal-Hansen, 1995). We and others have recently demonstrated that one of the MMPs gene, the chicken 72 kDa type IV collagenase, is expressed in early development (Yang *et al.*, 1996) and that its expression is particularly abundant in the dermis and feather buds of chicken at later stages of development (N.T. and G.B. unpublished data). To understand the effect of hydrocortisone on feather morphogenesis, we first investigated the expression pattern of c-*ets-1* during feather formation and compared it with the 72 kDa type IV collagenase. We show that hydrocortisone treatment of chicken embryos results in an increase of $p68^{c\text{-}ets\text{-}1}$ expression, and a decrease in p54 and 72 kDa type IV collagenase expression. Modulations of c-*ets-1* and 72 kDa type IV collagenase expression take place in the dermis and probably accompany concomitant disruption of the collagen fiber network.

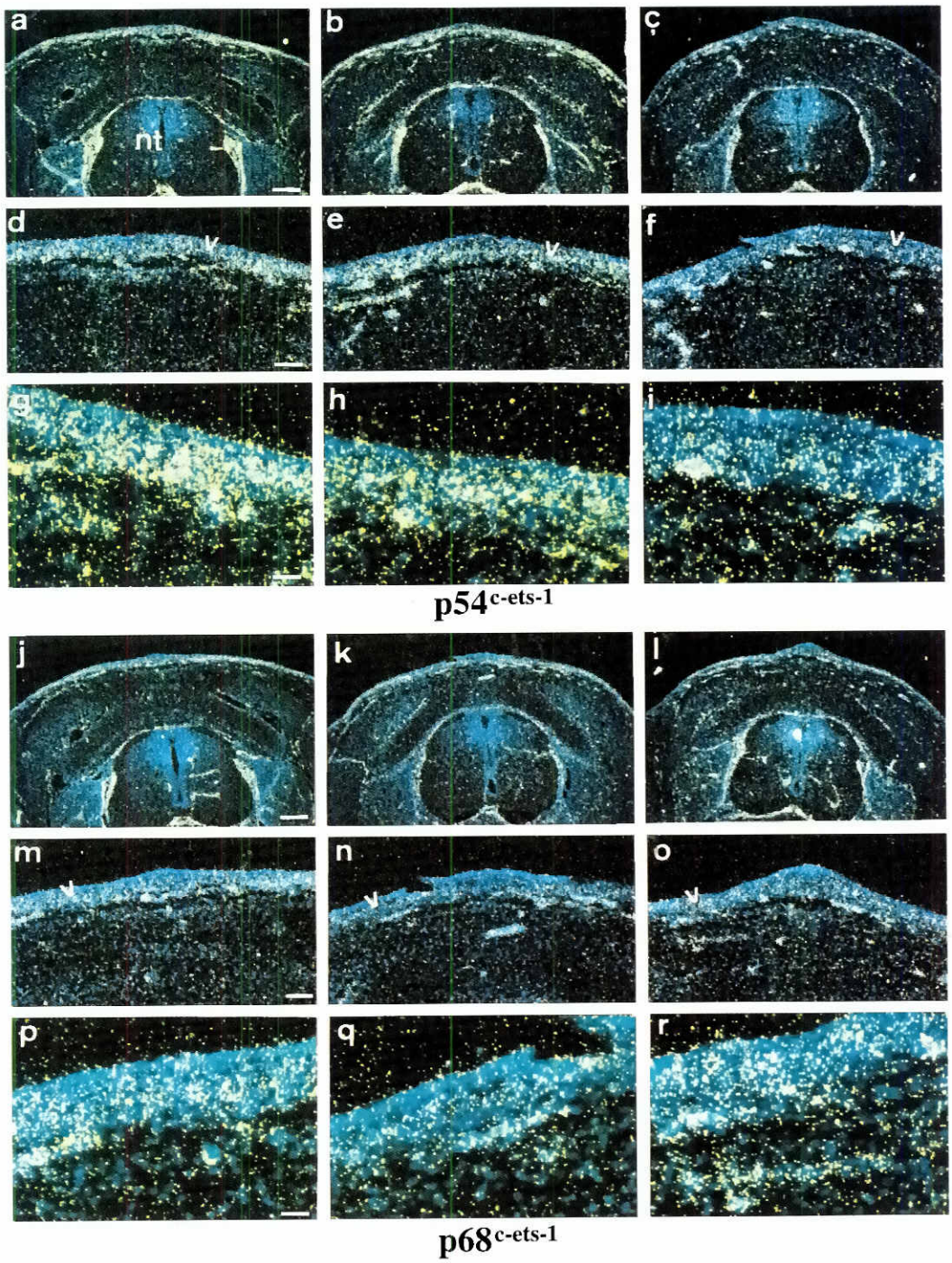

Fig. 2. Differential expression of p54 and p68 mRNA in the dermis of chicken embryos. *We used sense (not shown) and antisense mRNA probes for p54 and p68$^{c\text{-}ets\text{-}1}$ transcripts. The signal detected with the p54 probe in the dermis at E6.5* **(a,d and g)** *and 8 h later* **(b,e and h)** *was reduced in hydrocortisone-treated embryos (arrowhead)* **(c,f and i)**. *In contrast, the signal detected with the p68 probe in dermis at E6.5* **(j,m and p)** *and 8 h later* **(k,n and q)** *was increased in the hydrocortisone-treated embryos (arrowhead)* **(l,o and r)**. *nt, neural tube. Bars: g,h,i,p,q,r, 35 μm; d,e,f,m, n,o, 75 μm; a,b,c,j,k,l, 190 μm.*

Results

Hydrocortisone modulates c-ets-1 mRNA expression in chicken dermis

Transverse sections of chicken embryos at E 6.5 (Fig. 1a and d) and control embryos treated with PBS (Fig. 1b and e) or hydrocortisone (Fig. 1c and f) for 8 h were hybridized with a ^{35}S c-*ets-1* antisense probe recognizing both p54$^{c\text{-}ets\text{-}1}$ and p68$^{c\text{-}ets\text{-}1}$ encoding mRNA. As shown in Figure 1a,d,b and e, c-*ets-1* was expressed in normal dermis confirming previous results (Desbiens *et al.*, 1991; Quéva *et al.*, 1993). In hydrocortisone-treated chicken embryos, c-*ets-1* expression clearly decreased in dermis (Fig. 1c and f). Weak expression persisted in the neural tube and vessels of the hypoderm and those lining the vertebral arches. Thus, in different tissues expressing c-*ets-1*, hydrocortisone treatment resulted in a decrease of c-*ets-1* mRNA.

We were concerned that the decrease of c-*ets-1* expression could be a result of a general repression effect due to the hydrocor-

tisone treatment. Thus, in control experiments, adjacent sections of normal and treated chicken embryos were hybridized with a different probe such as that of *jun*. The dermis of control embryos showed a widespread c-*jun* expression (Fig. 1g and j). A similar pattern was found 8 h after PBS treatment (Fig. 1h and k). In contrast, in hydrocortisone-treated embryos, c-*jun* expression was significantly increased (Fig. 1i and l). This indicates that dermal cells responded to glucocorticoids by an increase in c-jun mRNA content. The same results were obtained in embryos treated for 24 h (not shown). We also observed a characteristic labeling in the spinal motoneurons of the neural tube (Fig. 1j and k) that remained unchanged after hydrocortisone treatment (Fig. 1l).

Hydrocortisone decreases p54 and increases p68 mRNA expression within the dermis

The chicken c-*ets-1* encodes, by alternate promoter usage, two nuclear proteins, p68 and p54. Therefore, we investigated the expression of the two c-*ets-1* transcripts.

The p54 transcripts were homogeneously distributed whereas the p68 transcripts were found only in a subpopulation of the dermal cells, also confirming previous results (Quéva *et al.* 1993). The amount of p54 transcripts, well expressed in the dermis of control chicken with (Fig. 2b,e and h) and without PBS (Fig. 2a,d and g) decreased 8 h after hydrocortisone treatment (Fig. 2c,f and i). Decreased amounts of p54 transcripts were observed all over the dermis (compare Fig. 2h and i). P54 expression also decreased in neural tube and in the few blood vessels still present in the hypodermis (compare Fig. 2b and c). Conversely, the p68 transcripts appeared apparently unchanged in treated dermis (compare Fig. 2m-n and o). The p68 signal was more diffuse in the treated dermis than in control tissue where p68 expression followed a dotted pattern, restricted to some groups of cells. Hydrocortisone treatment seems to recruit more cells, but does not increase the signal in each expressing cell.

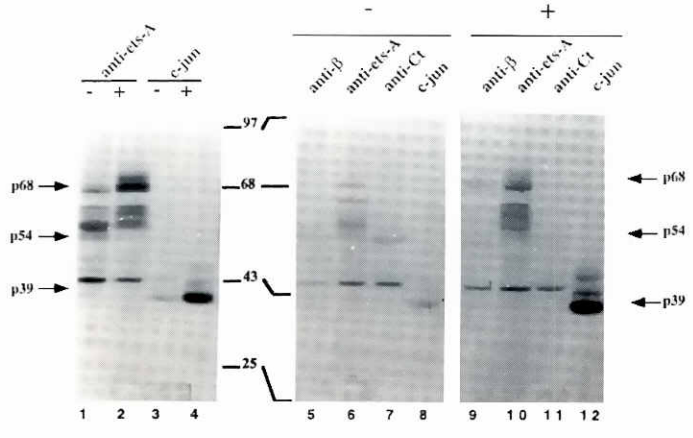

Fig. 3. Immunoprecipitation of Ets-1 and Jun from chicken dermis. *Chicken embryos were injected with 100 µl of PBS or hydrocortisone (0.1 mg in 100 µl of PBS). After 8 h of treatment, pieces of skin were pealed off the back of control embryos (-) and treated embryos (+). P39$^{c\text{-}jun}$ was immunoprecipitated with antiserum Ab-1* **(lanes 3,4,8 and 12)**. *The anti-ß serum recognizes p68$^{c\text{-}ets\text{-}1}$* **(lanes 5 and 9)**. *The anti-ets-A serum recognizes Ets-1 and Ets-2 proteins* **(lanes 1,2,6 and 10)**. *The antiserum anti-Ct recognizes p54$^{c\text{-}ets\text{-}1}$ and p68$^{c\text{-}ets\text{-}1}$ proteins* **(lanes 7 and 11)**. *The arrows indicate the p39, p54$^{c\text{-}ets\text{-}1}$ and p68$^{c\text{-}ets\text{-}1}$ bands.*

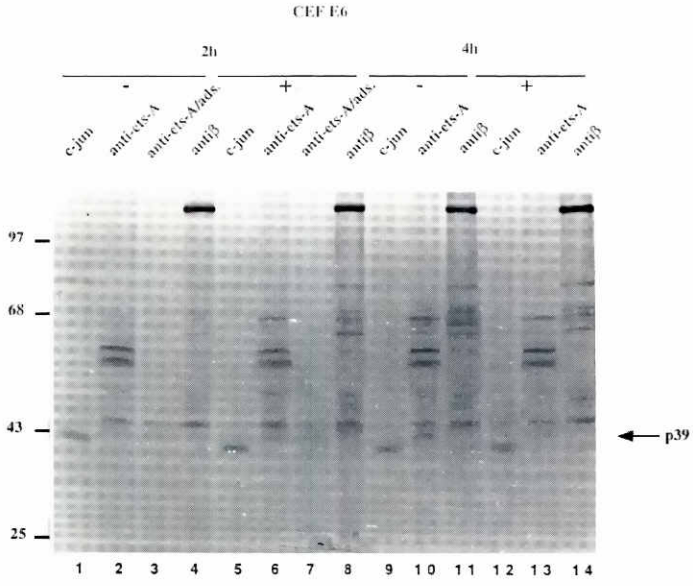

Fig. 4. Immunoprecipitation of Ets1 and Jun from cultured skin fibroblasts. *Cells from pieces of skin, pealed off the back of chicken embryos at E6, were cultured for 5 passages. Protein extracts from control cells* **(lanes 1 to 4 and 9 to 11)** *and cells treated with dexamethasone for 2 h* **(lanes 1 to 8)** *and 4 h* **(lanes 9 to 14)** *were immunoprecipitated with anti serum Ab-1 against the p39$^{c\text{-}jun}$* **(lanes 1,5,9 and 12),** *anti-ß serum against the p68$^{c\text{-}ets\text{-}1}$* **(lanes 4,8,11 and 14)** *and anti-ets-A serum* **(lanes 2,6,10 and 13)** *and its pre-immune against the Ets proteins* **(lanes 3 and 7)**.

Hydrocortisone modulates p54$^{c\text{-}ets\text{-}1}$ and p68$^{c\text{-}ets\text{-}1}$ protein expression in dermis

Next, it was interesting to determine whether the same modulation of c-ets-1 transcripts observed by *in situ* hybridization was observed at the protein level *in vivo* as well as *in vitro*. Protein extracts were prepared from skin pieces pealed off from the back of chicken control and hydrocortisone treated-embryos. Specific proteins were then immunoprecipitated with antibodies anti-EtsA, anti-ß and anti-Ct (see Materials and Methods).

The antisera anti-ets-A and anti-ß immunoprecipitated p68$^{c\text{-}ets\text{-}1}$ protein preferentially in the treated dermis 8 h after treatment. In contrast, less p54 was detected in treated dermis than in normal dermis (Fig. 3 lanes 1 and 2; 7 and 9). Thus, in line with the *in situ* hybridization experiments, hydrocortisone treatment caused a reduction of p54 at protein level. Similarly, the increased amount of p68 transcripts was reflected at the protein level (Fig. 3 lanes 1 and 2; 5 and 9; 6 and 10). In control experiments, immunoprecipitations performed with antibodies against c-Jun showed an increased amount of p39 in treated tissues compared to the control (Fig. 3 lanes 3 and 4; 8 and 12).

Surprisingly, when chicken skin cells were grown in culture, no modulation of c-Jun (Fig. 4 lanes 1,5,9 and 12) or c-Ets protein expression was detected (Fig. 4 lanes 2-4, 6-8, 10-11 and 13-14). This indicates that glucocorticoid effects on c-*jun* and c-*ets-1* expression are specific to a peculiar population of competent dermal cells only found in a definite spatio-temporal context.

The expression of the 72 kDa type IV collagenase in dermis is decreased by hydrocortisone

Transverse sections of control chicken embryos at E6.5 and E7 and embryos treated with hydrocortisone for 8 h and 24 h were hybridized with an antisense ^{35}S RNA probe specific to the 72 kDa type IV collagenase gene. As shown in Figure 4a and c, the 72 kDa type IV collagenase was widely expressed in the dermis of E6.5 and E7 chick, while the epidermis was negative. In treated embryos, 72 kDa type IV collagenase expression decreased in the dermis (Fig. 5b and d-f) although weak expression persisted in the feather buds which managed to grow under hydrocortisone treatment. Taken together, these results indicate that glucocorticoids decrease 72 kDa type IV collagenase expression in correlation with the $p54^{c\text{-}ets\text{-}1}$ expression.

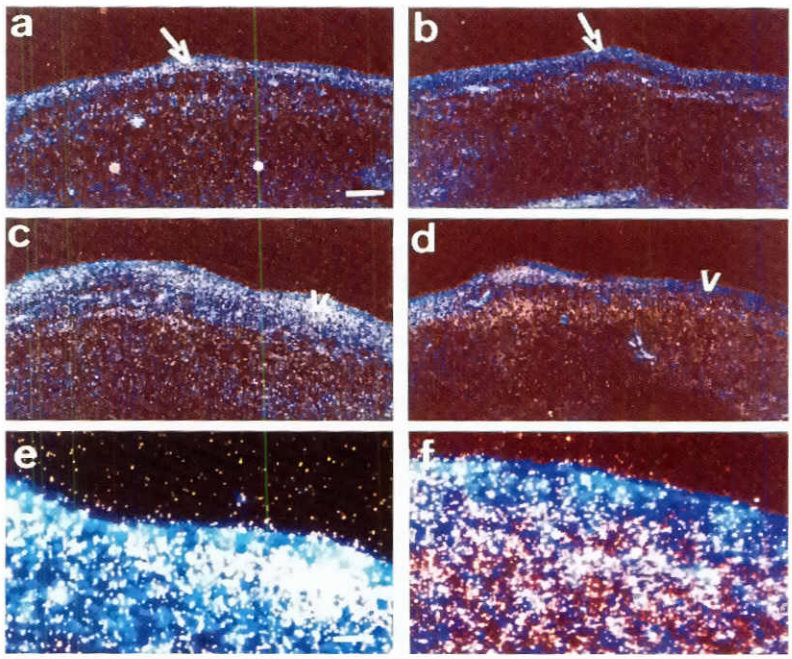

Fig. 5. Expression of 72 kDa type IV collagenase transcripts in the dermis of chicken embryos. *Chicken embryos were treated with PBS as control, or with 0.1 mg of hydrocortisone at E6.5 and fixed 8 h or 24 h after injection. Sections from dorsal dermis were hybridized with the 72 kDa type IV collagenase sense (not shown) and antisense mRNA probes. 72 kDa type IV collagenase is widely expressed in control dermis at E6.5+8 h or at E7* **(a,c and e)** *while its expression decreases in the dermis of hydrocortisone-treated embryos (arrowhead) at E6.5+8 h or at E7* **(b,d and f).** *We noted that 72 kDa type IV collagenase was still expressed in a feather bud which overcame hydrocortisone treatment and therefore managed to grow as previously described (Desbiens et al., 1992). Bars: e,f, 35 μm; a,b,d, 75 mm.*

Discussion

Hydrocortisone can partially or totally inhibit the normal development of feathers in the chicken embryos (Sengel and Züst, 1968; Züst, 1971; Stuart *et al.*, 1972). In the present study, we show that hydrocortisone treatments dramatically decrease $p54^{c\text{-}ets\text{-}1}$ levels while they increase both $p68^{c\text{-}ets\text{-}1}$ and c-jun expression. Furthermore, we found that the expression of $p54^{c\text{-}ets1}$ correlates with that of the matrix-metalloproteinase 72 kDa type IV collagenase. Taken together, our observations raise the question of how glucocorticoids modulate collagen synthesis, degradation or both, and whether c-*ets-1* takes part in this regulation.

A first hypothesis is that collagen accumulation results from increased synthesis. It has been reported that hydrocortisone increased mRNA levels of type I and V collagen genes in fibroblasts from human fetal dermis (Russell *et al.*, 1989). It is interesting that the promoter of the α1 chain of the type I collagen contains putative binding sites for c-ets-1 (EBS) (Bornstein *et al.*, 1987). However, whether c-*ets-1* plays a role in the collagen gene transcriptional regulation remains to be explored. It is worth noting that an alternative usage of c-*ets-1* promoters gives rise to two transcripts encoding two distinct proteins (Crepieux *et al.*, 1993). Therefore, it was necessary to determine which transcript was down-regulated. *In situ* hybridization experiments with specific probes allowed us to show that hydrocortisone modulates *in vivo* the p54 and p68 messengers in chicken dermis. We observed that the more abundant transcript, the p54 encoding messenger, strongly decreased in treated chicken dermis, and that the less abundant p68 transcript, in contrast, appeared to increase in treated dermis. Similar results were also observed at the protein level: the amount of p68 was increased and p54 became undetectable after treatment. This is interesting because the two c-ets-1 proteins have different transactivation domains and transactivating properties (Wasylyk *et al.*, 1991). $P68^{c\text{-}ets\text{-}1}$ was shown to be a more potent activator of the rat stromelysin promoter than $p54^{c\text{-}ets\text{-}1}$ (Wasylyk *et al.*, 1991). We have shown in this report that the expression of $p54^{c\text{-}ets1}$ decreases at mRNA and protein level in treated chicken dermis. Similarly, 72 kDa type IV collagenase expression decreased in hydrocortisone treated dermis. Therefore, it is tempting to speculate that $p54^{c\text{-}ets\text{-}1}$ could specifically regulate the 72 kDa type IV collagenase gene and the $p68^{c\text{-}ets\text{-}1}$ the collagen gene.

A second hypothesis would link collagen accumulation to an impaired degradation by endogenous collagenase. It has been shown that the human collagenase1 and stromelysin1 promoters are activated by two Ets-family members, Ets1 and Ets2, via functional EBS (Ets-binding sites) (Gutman and Wasylyk, 1990; Buttice and Kurkinen, 1993). Recently, it was demonstrated that different members of the Ets-family can differentially regulate collagenase1 and stromelysin1 gene activity, in an additive or antagonistic fashion (Buttice *et al.*, 1996). This suggests that probably there is a specific regulation of the matrix-metalloproteinases by the different Ets-family members. It is intriguing that we find a correlation of expression between the $p54^{c\text{-}ets\text{-}1}$ and the 72 kDa type IV collagenase, suggesting that the p54, but not the $p68^{c\text{-}ets\text{-}1}$, might be involved in the regulation of 72 kDa type IV collagenase. It will be interesting to find out whether the chicken promoter of the 72 kDa type IV collagenase contains functional EBS and is inducible by the $p54^{c\text{-}ets\text{-}1}$ product. However, we should note that although several matrix-metalloproteinases contain in their promoter region EBS and AP1 site, the human 72 kDa type IV collagenase promoter apparently lacks these binding motifs (Huhtala *et al.*, 1990; Gaire *et al.*, 1994). In addition, we cannot exclude that the decrease of 72 kDa type IV collagenase expression in hydrocortisone treated embryos is due to a different mechanism. For instance, glucocorticoids are known to inhibit matrix-metalloproteinases expression (Offringa *et al.*, 1988; Quinones *et al.*, 1989). The repression takes place at the AP1 site of the collagenase1 promoter via a physical interaction of

the glucocorticoid receptors with the Fos and Jun proteins (Jonat *et al.*, 1990; Lucibello *et al.*, 1990; Schule *et al.*, 1990). Interestingly, we found that c-*jun* was strongly expressed in control dermis and its expression increased at mRNA and protein level in the hydrocortisone treated dermis. Whatever the mechanism may be by which hydrocortisone regulates 72 kDa type IV collagenase, it is intriguing that hydrocortisone treatment of chicken embryos results in (1) inhibition of feather formation; (2) interstitial collagen accumulation; and (3), as observed here, a decreased 72 kDa type IV collagenase and $p54^{c\text{-}ets\text{-}1}$ expression. Therefore, it is possible that the collagen accumulation by hydrocortisone treatment is a result of a diminished collagenolytic activity due to decreased expression of 72 kDa type IV collagenase. This possibility is supported by recent results showing that 72 kDa type IV collagenase is able to cleave, in addition to type IV collagen and fibronectin, interstitial collagen with the same kinetics than that of the interstitial collagenase or collagenase 1 (Aimes and Quigley, 1995).

In conclusion we have found a link in the expression of a transcription factor, Ets1, and the matrix-metalloproteinases, 72 kDa type IV collagenase in the derma. Moreover we have pointed out that *in vivo* subtle mechanisms might take place to induce either the network of collagen fibers used by migrating cells in normal morphogenesis or under glucocorticoids treatments.

Materials and Methods

Biological model and tissue preparation

Eggs of the white Sussex chicken strain were incubated at 39°C in a humidified air chamber. E0 indicates the first day of incubation. At E6.5, chicken embryos were injected with 0.1 mg hydrocortisone (hydrocortisone 21 sodium succinate; Sigma) in 0.1 ml of PBS, whereas control embryos received 0.1 ml of PBS. The drug or control solutions were deposited on the chorioallantoic membrane at the level of the vascular area edge. Control and treated embryos were taken at E6.5, at E6.5+8 h and at E7.5. They were next fixed at 4°C for 16 h in 4% paraformaldehyde in PBS, dehydrated, embedded in paraffin and serially cut (7 µm). Transverse sections of the dorso-lumbar region were transferred to aminopropyltriethoxysilane (TESPA; Aldrich)-coated slides and incubated at 42°C for 2 days. Slides were stored at 4°C until use.

Probes

For c-ets-1 transcripts, we used a common probe derived from a 750-bp BglII-HindIII fragment of the E26 provirus (Leprince *et al.*, 1983). Antisense and sense RNA probes specific for the transcript encoding p54 were synthesized from a 140-bp EcoRI-ClaI fragment subcloned into pSP64 and pSP65 (Promega Biotech). This fragment contains the 5' untranslated region and exon I 54 of the $p54^{c\text{-}ets\text{-}1}$ (Duterque-Coquillaud *et al.*, 1988). Similarly, a 190-bp EcoRI-PstI fragment from $p68^{c\text{-}ets\text{-}1}$ cDNA encompassing exons a and b (Leprince *et al.*, 1988) was used as a template to synthesize the probe specific for the transcript encoding $p68^{c\text{-}ets1}$. The *jun* probe was synthesized from a 935-bp EcoRI fragment coming from the v-jun sequence (kindly provided by B. Wasylyk) and subcloned into pSP64. Specific RNA probes for the transcripts encoding p54 and p68 were transcribed from the digested plasmids using 200 mCi of α-^{35}S CTP and 200 mCi of α-^{35}S UTP. Riboprobes for jun and ets were labeled with 200 mCi α-^{35}S CTP only. The chicken 72 kDa type IV collagenase gene, kindly provided by M. Kurkinen (Yang *et al.*, 1996), was transcribed to produce sense and antisense probes and 200 mCi of α-^{35}S CTP were used for labeling.

In situ hybridization

The *in situ* hybridization protocol was a modification of the method of Cox *et al.* (1984) and was performed according to specific recommendations of Quéva *et al.* (1993). The best signal-to-background ratio was obtained at a concentration of 3 pg/ml for $p54^{c\text{-}ets1}$ and $p68^{c\text{-}ets1}$ specific probes and at temperatures of hybridization and washing of 70°C and 75°C, respectively. Three months of exposure were necessary to detect a significant signal with these two probes. On the other hand, hybridizations with the 750-bp c-ets-1 and jun probes required less stringent conditions: a probe concentration of 20 pg/ml, hybridization and washing at 60°C and 65°C respectively and two weeks of exposure. Slides were hybridized for 15-16 h, washed, dehydrated, dried and dipped in nuclear track emulsion (Kodak NTB2). After exposure and development, nuclei were stained with bisbenzimide (Hoescht dye 33258).

Immunoprecipitation

Treated and control dorso-lumbar skin was pealed off the back of embryos at E6.5, E6.5+8 h and E7.5. Pieces of skin were incubated for 2 h in the presence of 1 mCi L-^{35}S-methionine-cysteine (ICN, specific acivity of 1066 mCi.ml^{-1}) and centrifuged for 5 min at 1,000 rpm. Pellets were recovered in 1 ml of 1% SDS, 5% 2-mercaptoethanol, boiled for 5 min and diluted into 9 volumes of RIPA buffer (Radio immunoprotection assay). Equal amounts of radiolysates were immunoprecipitated with the commercial (Oncogene Science) polyclonal c-jun/AP-1 (Ab-1) antibody raised against amino acids 209 to 225, of the DNA binding domain in the C-terminal region of v-jun. Ab-1 reacts with the 39,000 dalton Jun protein. Antibodies raised against c-Ets-1: 1) a serum able to recognize both p54 and p68 Ets-1 proteins and Ets-2 (Ghysdael *et al.*, 1986) named anti-ets-A; 2) a serum specific of c-ets-1 raised against amino acids 473 to 485 of p68 which is a part of the carboxy-terminal of p54 and $p68^{c\text{-}ets1}$ (Quéva *et al.*, 1993) named anti-Ct; 3) a serum specific of $p68^{c\text{-}ets1}$ raised against a peptide corresponding to the ß region (Leprince *et al.*, 1990) named anti-ß.

Acknowledgments

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References

AIMES, R.T. and QUIGLEY, J.P. (1995). Matrix metalloproteinase-2 is an interstitial collagenase. *J. Biol. Chem. 270:* 5872-5876.

ALEXANDER, C.M. and WERB, Z. (1989). Proteinases and extracellular matrix remodeling. *Curr. Opin. Cell Biol. 1:* 974-979.

BIRKEDAL-HANSEN, H. (1995). Proteolytic remodeling of extracellular matrix. *Curr. Opin. Cell Biol. 7:* 728-735.

BORNSTEIN, P., McKAY, J., MORISHIMA, J.K., DEVARAAYALU, S. and GELINAS, E. (1987). Regulatory elements in the first intron contribute to transcriptional control of the human a1(I) collagen gene. *Proc. Natl. Acad. Sci. USA 84:* 8869-8873.

BUTTICÈ, G. and KURKINEN, M. (1993). A polyomavirus enhancer A-binding protein-3 site and Ets-2 protein have a major role in the 12-O-tetradecanoylphorbol-13-acetate response of the human stromelysin gene. *J. Biol. Chem. 268:* 7196-7204.

BUTTICÈ, G., DUTERQUE-COQUILLAUD, M., BASUYAUX, J.P., CARRERE, S., KURKINEN, M. and STEHELIN, D. (1996). Erg, an Ets-family member, differentially regulates human collagenase1 (MMP1) and stromelysin1 (MMP3) gene expression by physically interacting with the Fos/Jun complex. *Oncogene 13:* 2297-2306.

CHUONG, C.M. and EDELMAN, G.M. (1985). Expression of cell-adhesion molecules in embryonic induction. I. Morphogenesis of nestling feathers. *J. Cell Biol. 101:* 1009-1026.

COX, K.H., DE LEON, D.V., ANGERER, L.M. and ANGERER, R.C. (1984). Detection of mRNAs in sea urchin embryos by *in situ* hybridization using asymmetric RNA probes. *Dev. Biol. 101:* 485-502.

CREPIEUX, P., LEPRINCE, D., FLOURENS, A., ALBAGLI, O., FERREIRA, E. and STEHELIN, D. (1993). The two functionally distinct amino termini of chicken c-ets-1 products arise from alternative promoter usage. *Gene Expression 3:* 215-225.

DEMARCHEZ, M., MAUGER, A., HERBAGE, D. and SENGEL, P. (1984). Effect of hydrocortisone on skin development in the chick embryo: ultrastructural, immunohistological and biochemical analysis. *Dev. Biol. 106:* 15-25.

DESBIENS, X., QUEVA, C., JAFFREDO, T., STEHELIN, D. and VANDENBUNDER, B. (1991). The relationship between cell proliferation and the transcription of the nuclear oncogenes c-myc, c-myb and c-ets-1 during feather morphogenesis in the chick embryo. *Development 111:* 699-713.

DESBIENS, X., TURQUE, N. and VANDENBUNDER, B. (1992). Hydrocortisone perturbs the cell proliferation pattern during feather morphogenesis: evidence for disturbance of cephalocaudal orientation. *Int. J. Dev. Biol. 36:* 373-380.

DHOUAILLY, D. (1984). Specification of feather and scale patterns. In *Pattern Formation* (Eds. G.M. Malacinski and S.V. Bryant). Macmillan Publishing Company, New York, pp. 581-601.

DUTERQUE-COQUILLAUD, M., LEPRINCE, D., FLOURENS, A., HENRY, C., GHYSDAEL, J., DEBUIRE, B. and STEHELIN, D. (1988). Cloning and expression of chicken p54c-ets cDNAs: the first p54c-ets coding exon is located into the 40,0 kbp genomic domain unrelated to v-ets. *Oncogene Res. 2:* 335-344.

GAIRE, M., MAGBANUA, Z., McDONNELL, S., McNEIL, L., LOVETT, D.H. and MATRISIAN, L.M. (1994). Structure and expression of the human gene for the matrix metalloproteinase matrilysin. *J. Biol. Chem. 269:* 2032-2040.

GHYSDAEL, J., GEGONNE, A., POGNONEC, P., DERNIS, D., LEPRINCE, D. and STEHELIN, D. (1986). Identification and preferential expression in thymic and bursal lymphocytes of a c-ets oncogene-encoded Mr 54,000 cytoplasmic protein. *Proc. Natl. Acad. Sci. USA 83:* 1714-1718.

GUTMAN, A. and WASYLYK, B. (1990). The collagenase gene promoter contains a TPA and oncogene-responsive unit encompassing the PEA3 and AP-1 binding sites. *EMBO J. 9:* 2241-2246.

HUHTALA, P., CHOW, L.T. and TRYGGVASON, K. (1990). Structure of the human type IV collagenase gene. *J. Biol. Chem. 265:* 11077-11082.

JONAT, K., RAHMSDORF, H.J., PARK, K.K., CATO A.C.B., GEBEL, S., PONTA, H. and HERRLICH, P. (1990). Antitumor promotion and anti-inflammation down-modulation of AP-1 (Fos/jun) activity by glucocorticoid hormone. *Cell 62:* 1189-1204.

LEPRINCE, D., DUTERQUE-COQUILLAUD, M., LI, R.P., HENRY, C., FLOURENS, A., DEBUIRE, B. and STEHELIN, D. (1988). Alternative splicing within the Chicken c-ets-1 locus: implications for transduction within the E26 retrovirus of the c-ets proto-oncogene. *J. Virol. 62:* 3233-3241.

LEPRINCE, D., GEGONNE, A., COLL, J., DE TAISNE, C., SCHNEEBERGER, A., LAGROU, C. and STEHELIN, S. (1983). A putative second cell-derived oncogene of the avian leukaemia retrovirus E26. *Nature 306:* 395-397.

LEPRINCE, D., GESQUIERE, J.C. and STEHELIN, D. (1990). The chicken cellular progenitor of the v-ets oncogene, $p68^{c\text{-}ets\text{-}1}$, is a nuclear DNA-binding protein not expressed in lymphoid cells of the spleen. *Oncogene Res. 5:* 255-265.

LUCIBELLO, F.C., SLATER, E.P., JOOSS, K.U., BEATO, M. and MULLER, R. (1990). Mutual transrepression of Fos and the glucocorticoid receptor: involvement of a functional domain in Fos which is absent in FosB. *EMBO J. 9:* 2827-2834.

MACLEOD, K., LEPRINCE, D. and STEHELIN, D. (1992). The ets gene family. *Trends Biochem. Sci. 17:* 251-256.

MAUGER, A., DEMARCHEZ, M., HERBAGE, D., GRIMAUD, J.A., DRUGUET, M., HARTMANN, D. and SENGEL, P. (1982). Immunofluorescent localization of collagen types I and III, and of fibronectin during feather morphogenesis in the chick embryo. *Dev. Biol. 94:* 93-105.

OFFRINGA, R., SMITS, A.M.M., HOUWELING, A., BOS, J.L. and van der EB, A.J. (1988). Similar effects of adenovirus E1a and glucocorticoid hormones on the expression of the metalloprotease stromelysin. *Nucleic Acids Res. 16:* 10973-10984.

QUÉVA, C., LEPRINCE, D., STEHELIN, D. and VANDENBUNDER, B. (1993). $P54^{c\text{-}ets\text{-}1}$ and $p68^{c\text{-}ets\text{-}1}$, the two transcription factors encoded by the c-ets-1 locus, are differentially expressed during the development of the chick embryo. *Oncogene 8:* 2511-2520.

QUINONES, S., SAUS, J., OTANI, Y., HARRIS, J.E.D. and KURKINEN, M. (1989). Transcriptional regulation of human stromelysin. *J. Biol. Chem. 264:* 8339-8344.

RUSSELL, S.B., TRUPIN, J.S., MYERS, J.C., BROQUIST, A.H., SMITH, J.C., MYLES, M.E. and RUSSELL, J.D. (1989). Differential glucocorticoid regulation of collagen mRNAs in human dermal fibroblasts. *J. Biol. Chem. 264:* 13730-13735.

SCHMIDT, A., PELLEGRINO, R. and De CROMBRUGGHE, B. (1986). Transcriptional control of the mouse a2(I) collagen gene: functional deletion analysis of the promoter and evidence for cell-specific expression. *Mol. Cell. Biol. 6:* 347-354.

SCHNEIKERT J., LUTZ, Y. and WASYLYK, B. (1992). Two independent activation domains in c-ets-2 located in non-conserved sequences of the ets gene family. *Oncogene 7:* 249-256 .

SCHULE, R., RANGARAJAN, P., KLIEVER, S., RANSONE, L.J., BOLADO, J., YANG, N., VERMA, I.M. and EVANS, R.M. (1990). Functional antagonism between oncoprotein c-jun and the glucocorticoid receptor. *Cell 62:* 1217-1226.

SENGEL, P. (1957). Analyse en culture *in vitro* de l'activité du derme et de l'épiderme au cours de la croissance du germe plumaire de l'embryon de Poulet. *Bull. Soc. Zool. Fr. 82:* 233-238.

SENGEL, P. (1970). Morphogenesis of the skin and the cutaneous appendages in birds. In *Tissue Interactions During Organogenesis* (Ed. E. Wolff). Gordon and Breach Science Publishers, pp. 71-103.

SENGEL, P. (1990). Pattern formation in skin development. *Int. J. Dev. Biol. 34:* 33-50.

SENGEL, P. and RUSAOUËN, M. (1968). Aspects histologiques de la différenciation précoce des ébauches plumaires chez le poulet. *C.R. Hebd. Séanc. Acad. Sci. Paris 266:* 795-797.

SENGEL, P. and ZÜST, B. (1968). Malformations of plumage obtained by hydrocortisone injection into chick embryo. *C.R. Hebd. Séanc. Acad. Sci. Paris 267:* 1304-1307.

STUART, E.S., GARBER, B. and MOSCONA, A.A. (1972). An analysis of feather germ formation in the embryo and *in vitro*, in normal development and in skin treated with hydrocortisone. *J. Exp. Zool. 179:* 97-118.

WASYLYK, C., GUTMAN, A., NICHOLSON, R. and WASYLYK, B. (1991). C-ets-1 oncoprotein activates the stromelysin promoter through the same elements as several non-nuclear oncoproteins. *EMBO J. 10:* 1127-1134.

WESSELS, N.K. (1965). Morphology and proliferation during early feather development. *Dev. Biol. 12:* 131-153.

YANG, M., HAYASHI, K., HAYASHI, M., FUJII, J.T. and KURKINEN, M. (1996). Cloning and developmental expression of a membrane type-matrix metalloproteinase from chicken. *J. Biol. Chem. 271:* 25548-25554.

ZÜST, B. (1971). Le développement du plumage, d'après l'analyse des malformations cutanées produites par l'administration d'hydrocortisone à l'embryon de Poulet. *Ann. Embryol. Morphogen. 4:* 155-174.

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