

# Synthesis of Nerve Growth Factor mRNA in Cultures of Developing Mouse Whisker Pad, a Peripheral Target Tissue of Sensory Trigeminal Neurons

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**Abstract.** The developmental increase in the level of NGF mRNA in mouse maxillary process/whisker pad is paralleled *in vivo* by the biochemical and morphological differentiation of whisker pad epidermis, i.e., changes in the keratin expression pattern and the appearance of hair follicles. In cultures of maxillary processes, however, depending on the age of explanted tissue, the increase in NGF mRNA levels either precedes or follows the appearance of epithelial differentiation markers. In addition, we found that prevention of epithelial differentiation by retinoic acid did not affect the increase in NGF mRNA levels. Only in explants from E11.5 embryos was the timing of NGF mRNA production comparable to that of the *in vivo* situation,

whereas at earlier stages (E10/10.5) NGF mRNA levels increased slowly but never reached *in vivo* levels, even after extended culture periods.

However, the amount of NGF mRNA in E10/10.5 maxillary processes was strongly increased in the presence of medium conditioned by E11.5 explants. This effect was not mimicked by the factors IL-1 $\beta$  and TGF- $\beta$ 1 known to induce NGF mRNA in other systems. It is concluded that the developmental increase in NGF mRNA levels in developing mouse whisker pad is not linked to epidermal differentiation. Interestingly, it is strongly stimulated by a soluble factor(s) produced within the tissue.

NGF is essential for the survival of sensory and sympathetic neurons during development (5, 35). NGF is produced in the peripheral target fields of NGF-dependent neurons and the extent of neuron survival is controlled by the amount of NGF produced by a particular target. The onset of NGF responsiveness of developing sensory and sympathetic neurons and the timing of NGF production by peripheral targets has been analyzed in detail during embryogenesis. Neurogenesis and initial neurite outgrowth are not dependent on NGF and cannot be influenced by NGF (6, 9). In the mouse trigeminal system, where sensory neurons innervate facial skin, NGF dependence and NGF responsiveness is acquired at the time when the neurons innervate their target (6) and the production of NGF in this peripheral target starts when the earliest axons begin to reach the tissue (7). The onset of NGF production, however, is not elicited by ingrowing fibers, as NGF production in embryonic chick skin was observed even when innervation had been com-

pletely prevented (31). These studies suggested that the onset of NGF production during development is controlled by mechanisms intrinsic to the target tissue, but the nature of the factors involved remained unknown.

Factors which stimulate NGF expression in other cells and tissues have, however, been identified. The large increase in NGF mRNA levels in the rat sciatic nerve distal to a lesion (16) is due to interleukin-1 (IL-1)<sup>1</sup> released by invading macrophages (22). In cultures of astrocytes, NGF production is also strongly stimulated by IL-1 and TGF- $\beta$ 1, and similar effects are observed upon *in vivo* application of these factors (23, 33).

To investigate further the mechanisms that govern the onset of NGF production during development, we analyzed the control of NGF mRNA synthesis in a target of cutaneous sensory neurons of the trigeminal ganglion, the whisker pad. The whisker pad occupies the territory of maxillary and mandibular processes that merge around embryonic day 12 (E12). The maxillary process was chosen, since the timing of innervation, onset, localization, and extent of NGF production in this tissue is known from previous studies (6, 7). The epithelium of the maxillary process is reached by the earliest axons at E11. NGF mRNA synthesis in the maxillary process, localized in the epidermis, is first detectable at E10.5 and reaches a maximal level at E13. We have now used

1. *Abbreviation used in this paper:* IL-1, interleukin-1.

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cultures of maxillary processes to further investigate the developmental regulation of NGF production in this tissue. We found that the epidermis of cultured maxillary process explants, taken from mouse embryos as early as E10, is able to differentiate in vitro to a stage corresponding to E13 in vivo. Whereas in vivo NGF synthesis increases in parallel with epidermal differentiation, in vitro NGF mRNA production did not correlate with epidermal differentiation. The amount of NGF mRNA produced did, however, strongly depend on the age of the explant. In explants taken from E11.5 embryos, NGF mRNA levels rapidly increased, whereas the levels in E10/10.5 explant cultures remained low. However, NGF mRNA in E10/10.5 explants could be increased by culturing in medium that had been conditioned by maxillary processes of older (E11.5) embryos. Thus, NGF mRNA synthesis in developing whisker pad is not correlated with morphological differentiation and is controlled by soluble factor(s), which are distinct from the factors involved in the control of NGF synthesis in astrocytes and lesioned sciatic nerve (22, 23, 33).

## Materials and Methods

### Animals and Tissues

BALB/c albino mice were mated and females were examined for vaginal plugs, which was considered day 0 of embryonic development. The precise stage of development was determined by comparison of the external features of the embryos with the tables of Theiler (34). Maxillary processes (up to E12) and whisker pads were dissected with iridectomy scissors under a dissecting microscope.

### Tissue Culture

Maxillary processes/whisker pads were collected in PBS, washed in culture medium (Dulbecco's minimal essential medium, supplemented with 10% FCS), and placed at the edge of the culture membrane of Millicell-culture trays (Millicell, Millipore Corp., Bedford, MA) sitting in 24-well culture dishes (Costar Corp., Cambridge, MA). The volume of the culture medium was  $\sim 500 \mu\text{l}$ . Before use, the Millicell-culture trays were coated with acid-soluble collagen (type III from calf skin; Sigma Chem. Co., St. Louis, MO) followed by preincubation for at least 1 h with culture medium. Between 6 and 10 explants were cultured per tray and the amount of medium was adjusted so that the explants were at the air/medium interface, but still covered with medium. Follicular structures were observed to form perpendicular to the air/liquid interface and were absent in submerged culture conditions. Since the explants from younger embryos (E10/10.5) were difficult to orient with the epithelial side towards the air/medium interface, the number of follicles and the extent of the explant surface expressing the keratin pattern characteristic for differentiated epithelium was more variable than in older explants. The results of the quantitative analysis of morphological differentiation represent the mean of three independent experiments. In each experiment  $\sim 10$  explants were analyzed per time point.

In some experiments, human recombinant interleukin-1 $\beta$  (IL-1 $\beta$ ) (a generous gift from Dr. Gronenborn) was added at a concentration of 600 U/ml. TGF- $\beta$ 1 (from pig platelets; R&D Sys., Inc., Minneapolis, MN) and retinoic acid (all-trans; Sigma Chem. Co.) were added where indicated at a concentration of 10 ng/ml and  $10^{-6}$  M, respectively. All factors were applied after an initial culture period of 24 h by replacing culture medium by medium with added factors. To prevent degradation of added proteins, the protease inhibitors aprotinin (35 KIU/ml) and leupeptin ( $10^{-4}$  M) were added during treatments with TGF- $\beta$ 1 and IL-1 $\beta$ . TGF- $\beta$ 1 and IL-1 $\beta$  were biologically active as shown by stimulation of NGF mRNA synthesis in cultures of rat astrocytes. Conditioned medium from E11.5 explants was collected after 24 h in culture and kept at  $-20^\circ\text{C}$  until further use. For the analysis of NGF mRNA, cultured explants were frozen on dry ice and stored at  $-70^\circ\text{C}$ . For immunohistological analysis, explants were embedded in Tissuetek (Miles Inc., West Haven, CT) and kept at  $-70^\circ\text{C}$  until sectioning.

## Histology and Immunohistochemistry

For the immunohistochemical detection of cytokeratins, unfixed tissue was embedded in Tissuetek, frozen, sectioned, mounted on glass slides, and then fixed with acetone for 10 min at  $-20^\circ\text{C}$ . The sections were preincubated with PBS that was supplemented with 10% FCS. Sections were then either incubated overnight with rat monoclonal antibody Troma-1 (1:50 in PBS; a generous gift of Dr. R. Kemler, Freiburg) directed against keratin K8, or with mouse monoclonal antibody K4.8.60 directed against keratins K10/11 (1:50 in PBS; SIGMA Chemie, GmbH). The sections were washed three times with PBS, and then either incubated with rhodamin-coupled goat anti-mouse IgG (Dianova, Hamburg, FRG) or with biotinylated sheep anti-rat IgG (Amersham Corp., Arlington, IL), followed by FITC-coupled streptavidin. Sections were mounted in PBS/glycerin (1:1/vol:vol) and viewed with Leitz Orthoplan UV-optics. Fluorescence micrographs of different tissues were taken with identical exposure times and were processed identically through developing and magnification.

For the analysis of maxillary process/whisker pad differentiation explants were serially sectioned and alternate sections were stained for K8 and K10/11 keratins. The analysis of hair follicle formation was carried out on the same sections. Quantitative data are expressed as the mean  $\pm$  SD of at least three independent experiments. Each point per experiment represents the pool of  $\sim 10$  explants.

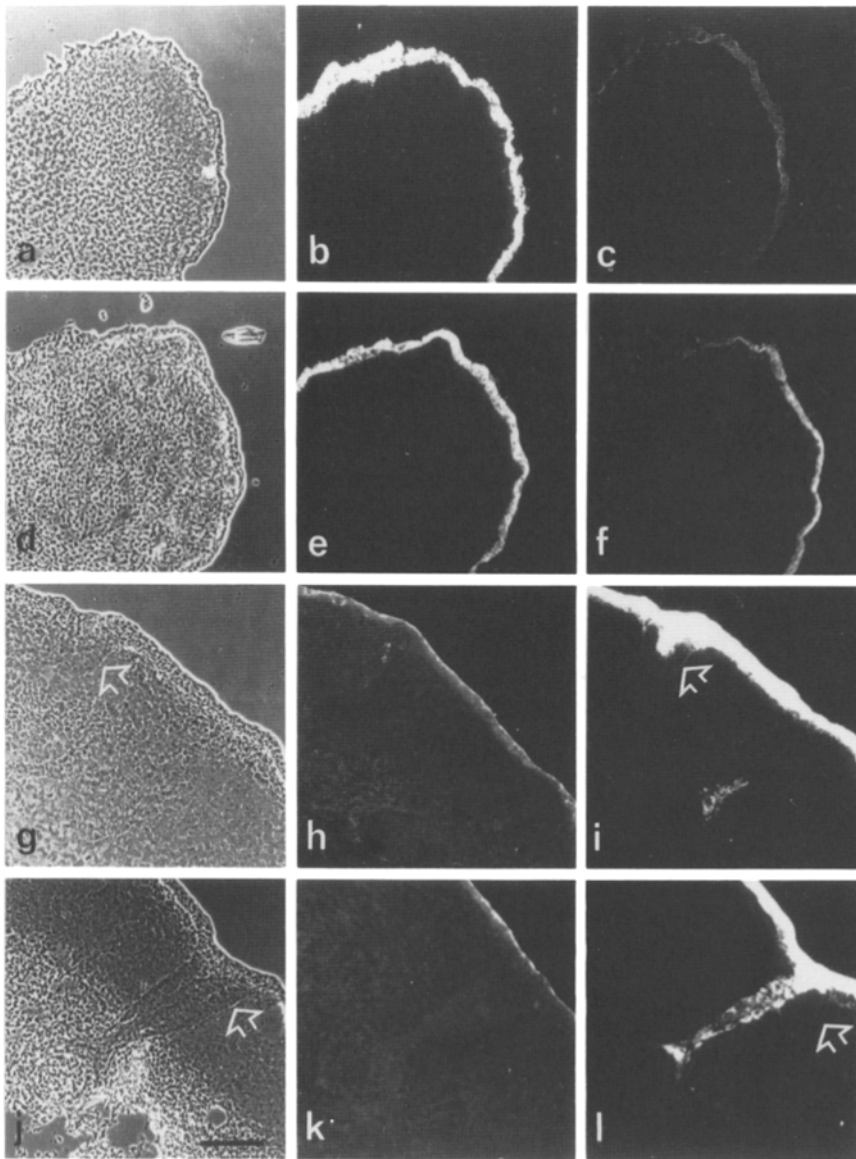
### NGF mRNA Quantification

The measurement of NGF mRNA in the maxillary process/whisker pad by quantitative Northern blot analysis was carried out essentially as described in detail previously (13, 14). For absolute quantification, a recovery standard (510 b) and a calibration standard (910 b) were coelectrophoresed in separate lanes. Hybridization was carried out at  $65^\circ\text{C}$  with  $5 \times 10^6$  cpm/ml of  $^{32}\text{P}$ -labeled NGF-cRNA-probe (0.9 kb) in 50% formamide. For each determination maxillary process/whisker pad had to be pooled from several animals. The number of maxillary processes/whisker pads required for each determination depended on the age of the embryos and amounted to  $\sim 25$  at E10/10.5, 18 at E11, 10 at E12, and 4 at E13.

## Results

### Epidermal Differentiation and Onset of NGF mRNA Synthesis in Developing Whisker Pad

The epidermis of the maxillary process, which becomes the major site of NGF mRNA synthesis in this tissue, differentiates initially during embryonic development from a primitive, two-layered epithelium to three-layer epidermis with hair follicles. To define more clearly the extent of epithelial differentiation in developing whisker pad, the expression of keratins was investigated. The presence of cytokeratins K8 and K10/11 as early and late markers for epithelial differentiation (10, 17, 25, 26) was analyzed immunocytochemically on tissue sections. Keratin K8 is strongly expressed in skin epithelia at E10, E11, and E12 (not shown), but then decreases to low levels at E13 and E14 and seems to be restricted to the periderm (Fig. 1). Keratins K10/11 first appear at E11, but the expression is restricted to a small region of the maxillary process and the staining intensity is low at E11 and E12 (not shown). From E13 onwards K10/11 are strongly expressed in the whisker pad epidermis, both in interfollicular keratinocytes and hair follicles. Interestingly, we found that the nasal epithelium displayed a different pattern of keratin expression; i.e., it was K8 positive and K10/11 negative during the time period analyzed (Fig. 2). By this criterion nasal epithelium could be identified in a small number of cultured maxillary process explants (see below). The presence of hair follicles is an additional characteristic of whisker pad epithelial differentiation. As described previously (1), hair follicles were not observed at E10. Primitive forms were ob-



**Figure 1.** Expression of keratins K8 and K10/11 in the epidermis of developing whisker pad. Alternate sections of maxillary process/whisker pad from E10 (a-c), E11 (d-f), E13 (g-i), and E14 (j-l) were stained for keratins K8 (b, e, h, and k) or K10/11 (c, f, i, and l) using specific monoclonal antibodies and FITC- or TRITC-labeled secondary antibodies. (Left column) Phase contrast; (middle and right columns) fluorescence. Arrows indicate follicular structures. Bar, 100  $\mu$ m. Please note that K10/11 keratin, which is a marker for complex epithelia, is only weakly expressed at E10, whereas keratin K8 is strongly expressed at E10 and E11.

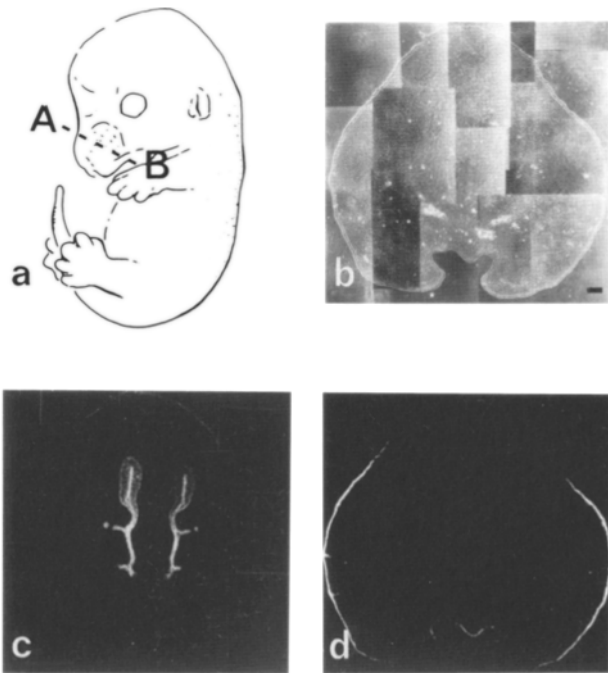
served in some animals at E11.5 and were consistently found at E12 and later.

The analysis of NGF mRNA expression by quantitative Northern blots demonstrated that NGF mRNA is not detectable at E10, but increases from very low levels at E10.5 up to a tissue content of  $\sim 0.6$  ng NGF mRNA/mg tissue at E13 (Fig. 3). A correlation between differentiation of whisker pad epithelium and NGF mRNA synthesis is evident in vivo—undifferentiated epithelium, which is characterized by the absence of keratins K10/11 and the lack of hair follicles, does not yet express NGF mRNA, whereas the increase in the expression of epithelial differentiation markers is paralleled by the increase in NGF mRNA levels. These results are in agreement with the previously published data of Davies et al. (7).

#### ***Epidermal Differentiation and NGF mRNA Synthesis in Cultured Mouse Prospective Whisker Pad***

To identify the factors and mechanisms involved in the control of NGF synthesis in mouse whisker pad, a culture sys-

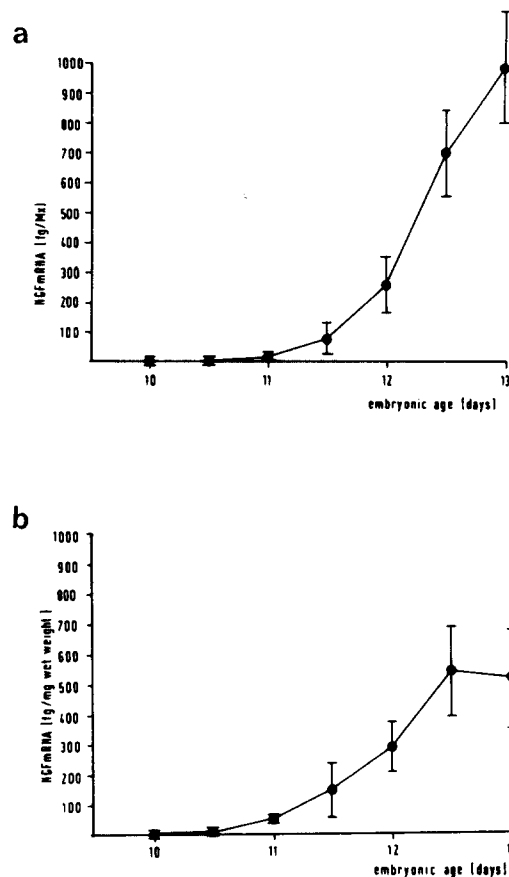
tem was developed that allows the differentiation of prospective whisker pad to be followed in vitro. Explants of maxillary process were cultured on a collagen-coated membrane in two-chamber culture dishes (see Materials and Methods). As suggested by previous studies (2, 3, 19), the explants were kept at the air/medium interface in order to permit epithelial differentiation. Under these culture conditions, the explants maintained a round, globular structure, and epithelial differentiation (as demonstrated by the appearance of follicular structures and by changing keratin pattern) increased synchronously (Fig. 4). Variations in the area of differentiated epithelium at a given time in culture may be due to the fact that a variable part of the area of the prospective whisker pad epithelium was exposed to the air/medium interface. For a quantitative evaluation of epithelial differentiation, explants were serially sectioned after different culture periods and analyzed for the expression of keratins K10/11 as late epithelial marker and for the number of hair follicles per explant. It was observed that the rate of epithelial differentiation depended strongly on the differentiation stage at which the tissues were dissected and put into culture.



**Figure 2.** Differential expression of keratins K8 and K10/11 in nasal and skin epithelia of E14 mouse embryos. Sagittal sections (line A–B) were cut in the region of the prospective whisker pad as illustrated in *a* and were then alternatively stained for keratin K8 (*c*) and keratin K10/11 (*d*). Whereas the early keratin K8 is no longer expressed in whisker pad epidermis (*c*), it is maintained in the simple epithelium of the nose. The late keratin K10/11 is absent in nasal epithelia but expressed in skin (*d*). (*b*) Phase contrast; (*c* and *d*) immunofluorescence. Bar, 100  $\mu\text{m}$ .

Maxillary processes from E11.5 embryos differentiated rapidly in culture (Fig. 5, *a* and *b*). Within 2 d in culture, keratins K10/11 were detectable in all explants; and within 4 d in culture, hair follicles were present in every explant. In contrast, a much longer time period was required for maxillary processes from E10 or E10.5 embryos to differentiate. After 4 d in culture, only 30% of the explants were K10/11 positive and hair follicles were still absent in all explants. It took another 4-d culture period until all E10 explants were positive for K10/11 and hair follicles. With respect to morphological and biochemical differentiation, the E10 and E10.5 explants displayed a virtually identical time course and for this reason, E10 and E10.5 explants were combined for the analysis of NGF mRNA expression.

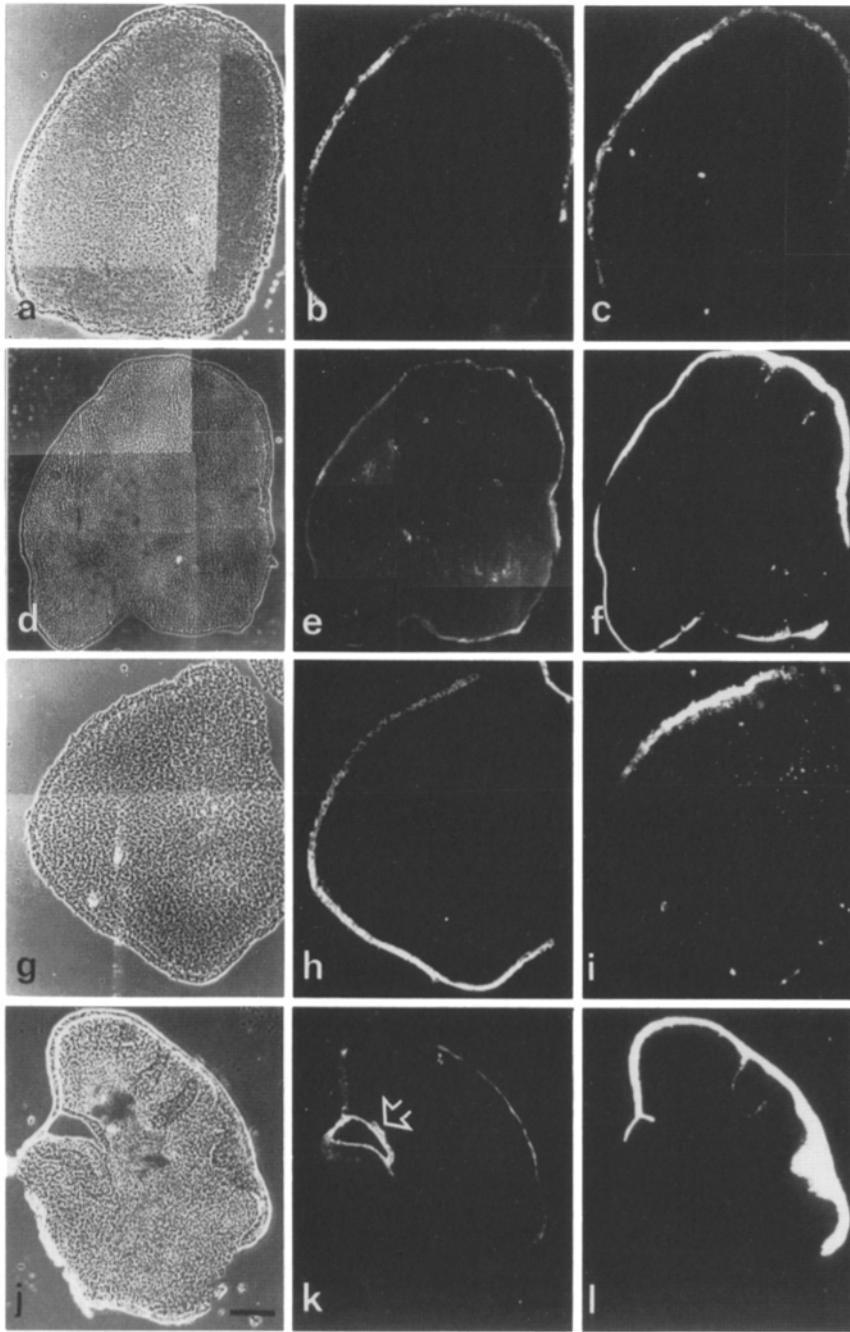
Also, the development of NGF mRNA expression in explant cultures showed a strong dependence on the initial differentiation stage of the explant (Fig. 6). Whereas the NGF mRNA level rapidly increased in cultures of E11.5 explants and reached an expression level comparable to the concentration present in vivo, only a very slow increase was observed in cultures of E11 and E10/10.5 explants. NGF mRNA levels in E11 explants were significantly lower than in E11.5 explants between 1 and 4 d in culture (Fig. 6). In E10/10.5 explants, the NGF mRNA levels were significantly lower than the in vivo levels even after 8 d in culture. Taken together, these data suggest that in vitro maxillary processes from E10 to E11 embryos differentiate, i.e., develop hair follicles and differentiated keratin expression pattern, but remain immature with respect to NGF mRNA expression.



**Figure 3.** Increase in NGF mRNA content during whisker pad development. NGF mRNA levels in maxillary process/whisker pad were determined by quantitative Northern blot hybridization and expressed as fg NGF mRNA per maxillary process (*a*) or per mg wet weight (*b*). The values represent the mean  $\pm$  SD of at least three independent experiments.

### Modulation of NGF mRNA Levels in Explant Cultures of Maxillary Process

**Effects of IL-1 $\beta$ , TGF- $\beta$ 1, and Retinoic Acid.** The culture system developed in the present study was used to identify factors that affect the regulation of NGF mRNA levels in a peripheral target of NGF-dependent sensory neurons. E10/10.5 explants were used to investigate the early onset of NGF production, and E11.5 explants to study effects of factors on already initiated NGF mRNA synthesis. NGF mRNA levels in explant cultures of rat sciatic nerve (22) and in cultured rat astrocytes (33) increase in the presence of IL-1. Astrocytes also respond to treatment with TGF- $\beta$ 1 with an increase in NGF mRNA both in vitro and in vivo (23). In contrast, we did not observe a stimulation of NGF mRNA synthesis when cultures of E11.5 maxillary processes were maintained in the presence of TGF-1 $\beta$  or IL-1 (Fig. 7). To exclude the possibility that ongoing NGF mRNA synthesis in E11.5 explants is already maximally stimulated, the factors were also tested in cultures of E10/10.5 explants. NGF mRNA levels are undetectable at the onset of culture and increase to low levels in these cultures and thus a stimulatory effect should be easily detectable. Effects of IL-1 and TGF- $\beta$ 1, however, were not observed after 6 or 24 h of treatment, neither on



**Figure 4.** Epidermal differentiation in cultured mouse maxillary process. E11.5 maxillary processes were analyzed after 2 (*a-c*) and 4 d (*d-f*) in culture for the expression of keratins K8 (*b* and *e*) and K10/11 (*c* and *f*). E10.5 maxillary processes were analyzed after 4 (*g-i*) and 8 d (*j-l*) in culture for the expression of keratins K8 (*h* and *k*) and K10/11 (*i* and *l*). (*a*, *d*, *g*, and *j*) Phase contrast; (*b*, *c*, *e*, *f*, *h*, *i*, *k*, and *l*) fluorescence. Please note that the rate of differentiation is delayed in cultures of E10.5 explants as compared with E11.5 explants. (cf. *e*, *f*, and *h*, *i*). However, in 8-d cultures hair follicles (*j*) and strong expression of keratins K10/11 (*l*) are apparent. (*Arrow*) Nasal epithelium has been included in this explant by accident. Bar, 100  $\mu$ m.

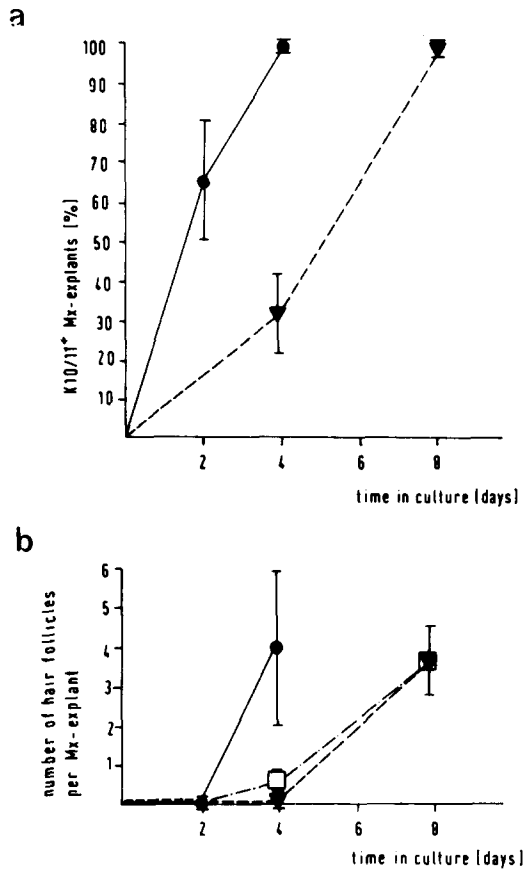
NGF mRNA levels nor on follicle formation. Under comparable conditions, increased NGF mRNA levels were observed in injured sciatic nerve 6 and 24 h after IL-1 $\beta$  treatment, and in astrocyte cultures also 6 and 24 h after TGF- $\beta$ 1 treatment (22, 23, 33).

The effects of vitamin A and its derivatives on epithelial differentiation is well known, and retinoic acid is thought to be the major biologically active form or to mimic the effects of endogenous skin retinoids (11, 18, 19, 30). Thus, it was of interest to determine the effects of retinoic acid on both morphological differentiation and NGF mRNA expression of developing whisker pad. Maxillary processes from E11.5 embryos treated with retinoic acid ( $10^{-6}$  M) for 24 or 72 h in culture, were strongly inhibited in their differentiation.

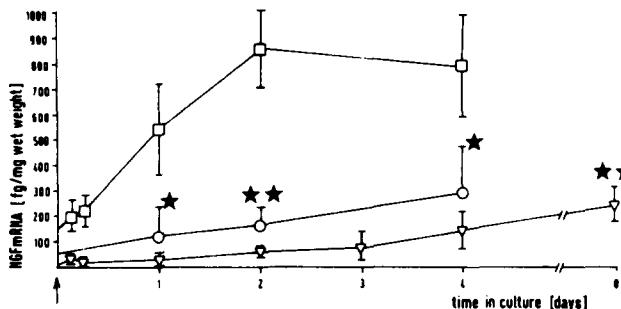
The expression of keratins K10/11 was much reduced in intensity and in the extent of the explant surface that was stained (Fig. 8). In addition, the number of hair follicles was decreased about fivefold. In spite of these strong effects of retinoic acid on morphological differentiation, NGF mRNA synthesis was not affected in either E11.5 or E10/10.5 explants after 6, 24, or 72 h of treatment with retinoic acid (Fig. 7).

The finding that retinoic acid affects epithelial differentiation, but not NGF mRNA levels in maxillary processes, provides additional evidence that in the developing whisker pad epithelium, morphological differentiation is not linked to NGF mRNA synthesis.

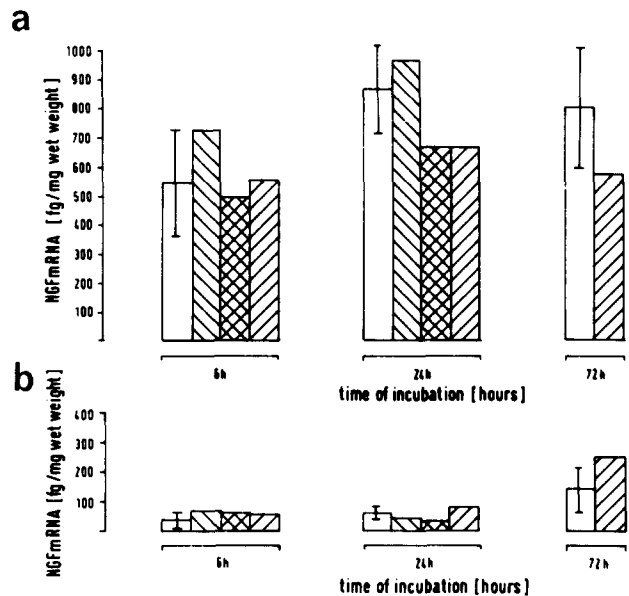
**Conditioned Medium Effects.** We found that the extent of



**Figure 5.** Quantitative analysis of epidermal differentiation of cultured mouse maxillary process. Maxillary processes from E11.5 (●), E10.5 (□), and E10 (▼) embryos were maintained in culture. After different culture periods the explants were serially sectioned and analyzed for the proportion of explants which express the late keratin marker K10/11 (a) and for the number of hair follicles (b). The values represent the mean of three independent experiments and for each experimental point ~10 explants were analyzed.



**Figure 6.** Time-dependent increase in NGF mRNA levels in cultured maxillary process. Maxillary processes from E10/10.5 (▽), E11 (○), and E11.5 (□) embryos were cultured and analyzed after different time periods (3 h, 6 h, 1 d, 2 d, 3 d, 4 d, and 8 d) for the amount of NGF mRNA expressed in the explants by quantitative Northern blots. The values represent the mean  $\pm$  SD of three independent experiments. The NGF mRNA content of E11 explants is significantly lower than the levels in E11.5 explants at the corresponding culture period. Even after 8 d in culture, NGF mRNA levels of E10/10.5 explants are significantly lower than the levels of E11.5 explants after 4 d in culture. (\*  $P < 0.01$ ; \*\*  $P < 0.0003$ ).



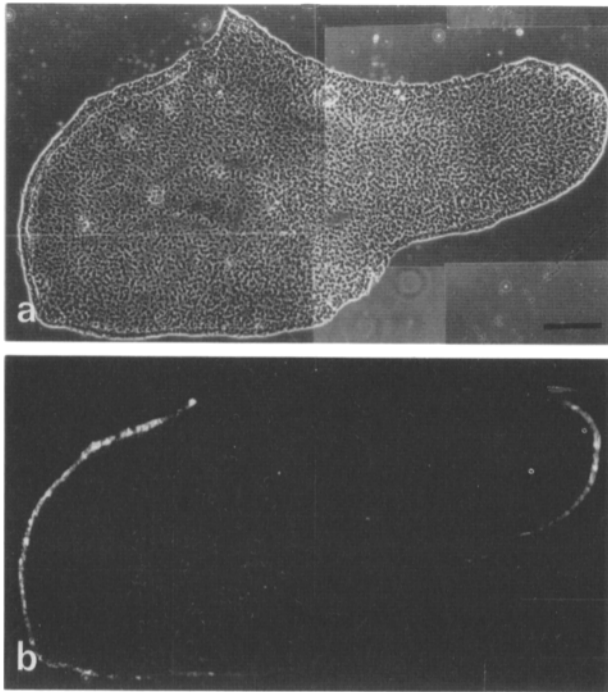
**Figure 7.** Effect of IL-1, TGF-1 $\beta$ , and retinoic acid on NGF mRNA levels in cultured maxillary process. Cultures of E11.5 (a) or E10/10.5 (b) maxillary processes were treated after 1 d in culture for the indicated times with TGF $\beta$ 1 (▨), IL-1 $\beta$  (▩), retinoic acid (▧), or control medium (□), and then analyzed for the levels of NGF mRNA by quantitative Northern blots. Control values are expressed as mean  $\pm$  SD of three determinations and values for the treated culture reflect the mean of two determinations.

NGF mRNA synthesis in culture depended on the differentiation stage of the embryo. The strong increase of NGF mRNA levels in E11.5 explants as compared to E10/10.5 explants may be explained by the presence of soluble induction factors which are absent at E10/10.5, and are not expressed in E10/10.5 explants with time in culture. To test this hypothesis, medium conditioned by E11.5 explants was added to E10/10.5 explants. A slight increase of NGF mRNA levels was already detectable after 1 d in culture. After a culture period of 4 d in E11.5-conditioned medium, NGF mRNA levels in E10/10.5 explants reached values observed in vivo in E13 whisker pads and were about threefold higher than the controls (Fig. 9).

## Discussion

The survival of peripheral neurons is under the control of neurotrophic factors present in peripheral and central targets. During development, neurotrophic factor production in the target has been shown to be coordinated with the onset of neuronal responsiveness to neurotrophic factors. However, the developmental expression of these factors and their receptors on receptive neurons occurs in the absence of any interaction between neurons and target, and thus seems to require independent timing mechanisms (31, 36).

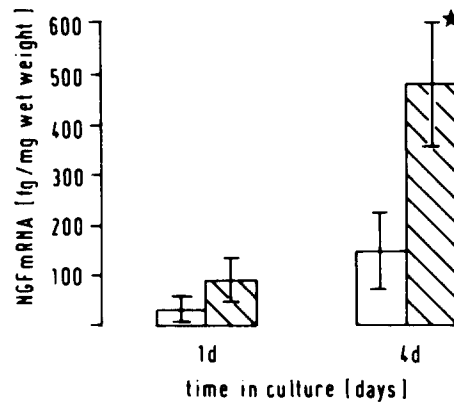
In the present paper, we have investigated in vivo and in vitro the onset of NGF mRNA expression in a target of NGF-dependent neurons, the embryonic mouse whisker pad. NGF mRNA in this tissue increases from undetectable low levels at E10 to maximal levels at E13 (4, 7) and this increase correlates with epithelial differentiation, i.e., changes in keratin



**Figure 8.** Retinoic acid treatment interferes with epithelial differentiation of maxillary process. Cultures of E11.5 maxillary processes were treated after 24 h in culture with  $10^{-6}$  M retinoic acid for 24 and 72 h in culture, and then analyzed for the expression of keratins K10/K11. Please note the absence of hair follicles (a) and the reduced expression of the late keratins (b) (cf. Fig. 4). Bar, 100  $\mu$ m.

pattern and appearance of hair follicles. The *in vitro* studies demonstrated, however, that NGF mRNA expression is not linked to epithelial differentiation. The onset of NGF mRNA production seems rather to be controlled by a soluble factor(s) produced by the tissue and which can be detected in conditioned medium of E11.5 explants, but which is not produced when E10/10.5 explants are brought into culture.

The majority of NGF mRNA expressed in mouse whisker pad is present in surface and follicular epithelium (4, 7). Thus it seemed of interest to define the differentiation status of whisker pad epidermis during the onset and increase of NGF mRNA synthesis. Mammalian epidermis consists initially of a two-layered epithelium—an inner layer of embryonic basal cells and an outer layer, the periderm. Stratification into a complex epithelium consisting of basal, peridermal, and intermediate cell layers is associated with the formation of primary hair follicles and changes in the cytokeratin pattern. Simple one- or two-layered epithelia express keratins K8/K18, whereas keratins K10/K11 appear during the formation of complex, multilayered epithelia (25, 26). The surface epithelium of the prospective mouse whisker pad strongly expresses keratin K8 from E10 up to E12. The expression of K8 disappears in whisker pad epidermis around E13. It remains, however, expressed in other epithelia like nasal and oral epithelia (17). The marker for complex epithelia was first found at E11 but its expression was restricted to a small area of the maxillary primordium. Around E13 the expression of K10/K11 increased both with respect to the intensity and the surface area where it could be detected in whisker pad. K10/K11 were not detected in nasal epithelium at that age. The formation of multilayered



**Figure 9.** Medium conditioned by E11.5 whisker pad increases NGF mRNA levels in cultures of E10/10.5 maxillary process. E10/10.5 maxillary process was cultured for the indicated time period in the presence of medium conditioned by E11.5 whisker pad explants (▨), or with control medium (□), and were analyzed for the levels of NGF mRNA. After 4 d in culture with conditioned medium, the NGF mRNA levels are significantly increased (\*  $P < 0.001$ ) above control cultures. Values are given as the mean of three independent determinations.

epidermis is a prerequisite for the development of hair follicles. In agreement with the analysis of cytokeratin expression, we consistently observed hair follicles at E12, as described previously (1). Both sets of data, keratin pattern and follicle formation, localized the transition from simple to complex epithelia to the time period between E11 and E12.

NGF mRNA can be detected in whisker pad as early as E10.5 by Northern blot analysis, but the levels remain low until E11. The major *in vivo* increase of NGF mRNA synthesis between E11 and E12.5 is paralleled by an increased expression of cytokeratins K10/K11 and hair follicles. The parallel increase of NGF mRNA expression and epidermal differentiation raised the possibility that both phenomena are correlated and even causally linked, for instance by common inducing factors or signal transduction mechanisms.

To address this question and to investigate further the control of NGF mRNA synthesis in whisker pad, a culture system had to be developed which supports epithelial differentiation. Keratinocyte differentiation in serum-containing medium occurs only when the cells are positioned at the air/medium interface which is assumed to provide an optimal concentration or diffusion gradient of retinoids present in serum (3, 11, 18) and which is thought to reflect the *in vivo* situation of skin epithelia. Explants rather than dissociated keratinocytes were chosen in the present study to enable dermal-epidermal interactions and the formation of hair follicles. The culture conditions supported epithelial differentiation and allowed the examination of this process in a reproducible and quantitative manner. All explants of maxillary process differentiated in culture and eventually displayed the characteristics of E13 whisker pads, i.e., hair follicles and high levels of keratins K10/K11. However, the rate of differentiation did not correspond to the *in vivo* situation. E11.5 explants, which take 2 d to differentiate *in vivo*, were positive for keratins K10/K11 after 4 d *in vitro*. E10/E10.5 explants reached the differentiation stage of E13 whisker pad only after 8 d in culture. It is likely that the slow differentiation is due to suboptimal conditions for the early onset of

differentiation. A retardation of epidermis stratification could for instance result in delayed formation of hair follicles. E11.5 explants, where stratification has already started at the beginning of the culture, would be affected much less than E10/E10.5 explants. It should also be pointed out that the culture system provided favorable conditions for differentiation, but apparently not for the growth of the explant. This resulted in a constant volume and surface area of the explant and it is assumed that for this reason follicles form in much smaller numbers than in vivo.

The correlation observed in vivo between whisker pad epithelial differentiation and NGF mRNA expression suggested that a similar correlation would also be maintained in vitro. This was however not observed. In E11.5 explants, NGF mRNA levels increased with a similar time course as in vivo and thus the NGF mRNA increase preceded epithelial differentiation, reflected by changed cytokeratin pattern. Maximal NGF mRNA levels, reached after 2 d, were higher than the levels observed in vivo. This may also be due to the limited growth of the explants, resulting in an increased proportion of epidermis to mesenchyme, containing high and low NGF mRNA levels, respectively. Whereas in E11.5 explants, the increase in NGF mRNA levels preceded the increase in differentiation markers, in E10/10.5 explants differentiation was completed after 8 d in culture, but NGF mRNA levels remained low even after this extended culture period.

Taken together, these results exclude a causal relationship between differentiation and NGF mRNA synthesis. This conclusion is supported by experiments where epidermal differentiation has been prevented by the application of retinoic acid. Vitamin A and its derivatives, the retinoids, are known to affect proliferation and differentiation of epithelial cells and retinoic acid has been shown previously to prevent the expression of the late cytokeratins K1 and K10/11 in epithelial cells and epidermis (11, 18, 19). In the present cultures we also observed a dramatic reduction in the expression of keratins K10/11 and a complete prevention of hair follicle formation. However, the expression of NGF mRNA was completely unaffected by this treatment.

The site of NGF mRNA synthesis in explant cultures is unclear and needs to be analyzed in further studies. However, since NGF mRNA expression in cultured maxillary processes of different ages seems to be determined by intrinsic, stage-specific cues, it seems likely that in vitro NGF mRNA is expressed in mesenchyme and epithelium as previously shown for the in vivo situation. The high levels of NGF mRNA in follicular epithelium are reminiscent of the localization of Merkel cells, the epithelial target of sensory neurons in mature whisker pad (28). Interestingly, Merkel cells produce NGF in vitro (37), suggesting that Merkel cells may be the source of epithelial NGF during development. However, the first presumptive Merkel cells can be distinguished by morphological criteria in rat whisker pad at E16 (8, 27). A role of Merkel cells in epithelial NGF mRNA synthesis thus would imply a large number of Merkel cell precursors that account for the onset of NGF mRNA synthesis at E10.5 and the uniform distribution of NGF mRNA in surface and follicular epithelium (7), a hypothesis that can only be tested when markers for Merkel cell precursors are available.

The factors which control differentiation and affect NGF mRNA synthesis are not known. The present in vitro study suggests however that NGF synthesis is independently regu-

lated from epithelial differentiation, and thus most likely different factors and mechanisms are involved.

The behavior of E10/10.5 explants with respect to NGF mRNA synthesis indicates that onset of NGF synthesis in maxillary process is possible in vitro, but that the subsequent increase observed during normal development requires further stimulation and supporting factors which do not occur in explants of that age. In contrast, in E11.5 explants, the timing of NGF mRNA expression corresponds to the in vivo situation. This seems to be due to the ability to make a diffusible factor(s), which can be released into the medium and which can stimulate NGF mRNA synthesis in E10/10.5 maxillary process. An important change must occur in the maxillary process between E10.5 and E11.5, and in view of the important role of mesenchyme for epithelial differentiation (32) this change may occur either in the epithelium or in the mesenchyme. It has been speculated previously that the onset of NGF mRNA synthesis in mouse maxillary process may be triggered by the nerve fibers growing in from the trigeminal ganglion at E11. Although the present data seem to support this hypothesis, we have previously excluded this possibility by showing that normal NGF mRNA levels are reached in limb skin which never becomes innervated (31) and it seems unlikely that different regions of skin have different regulatory mechanisms for NGF synthesis.

What is the molecular nature of the factor that is released from whisker pad and is able to stimulate NGF mRNA levels? Two factors, TGF- $\beta$ 1 and IL-1 $\beta$ , which stimulated NGF mRNA synthesis in sciatic nerve explants and cultured astrocytes (22, 23, 33), had no effect in the present system, although both factors have been implicated in skin development. Keratinocytes express IL-1 $\beta$  and also respond to IL-1 $\beta$  by increased proliferation (20). TGF- $\beta$ 1 protein was detected in mesenchyme surrounding developing hair follicles in E11 whisker pad (12) and TGF- $\beta$ 1 mRNA is only expressed in follicular, but not in interfollicular, areas of the epidermis and could not be observed before E12.5 (21). In another immunohistological study (29), TGF- $\beta$ 1, - $\beta$ 2, and - $\beta$ 3 were not detected before E14.5 in mouse whisker pad. Thus, neither the timing nor the localization of TGF- $\beta$ 1 expression in whisker pad correlates with NGF mRNA expression (4, 7). Together with the now-described absence of TGF- $\beta$ 1 effects on NGF mRNA expression, these data argue against a role for TGF- $\beta$ 1 in the regulation of NGF expression in developing mouse whisker pad. To definitely clarify the roles of IL-1 $\beta$  and TGF- $\beta$ 1 in the control of NGF mRNA synthesis in different tissues, experiments to eliminate those factors in vivo need to be done.

The present data support the conclusion that NGF expression can be regulated by a multitude of factors; there is no single control mechanism. This has also been exemplified by the recent finding that the two cell types present in the sciatic nerve, fibroblasts and Schwann cells, use different mechanisms and respond to different factors to control NGF mRNA expression (24).

It should be pointed out that also the time course of NGF mRNA expression in whisker pad differs from the time course of NGF mRNA expression in explant cultures of adult tissues. In sciatic nerve cultures (15, 16) and also in cultures of rat iris (13), NGF mRNA content increases rapidly within the first hours in culture. This initial, rapid increase was never observed in explants of maxillary process. There is evidence to indicate that the rapid, initial NGF mRNA in-



crease depends on the presence of lesioned axons in the cultured tissue (Meyer, M., and H. Thoenen, manuscript in preparation). Since the dissection of E11.5 maxillary process also results in the lesion of axons, it is suggested that either the axon numbers are still too low or that developing tissues differ from adult tissues in their response to axon lesion. The finding that the maxillary explants neither showed the rapid lesion-mediated NGF mRNA increase nor responded to IL-1 $\beta$ , which mediates the slow NGF mRNA increase in lesioned nerve, may indicate that different factors and mechanisms are involved in lesion-induced upregulation of NGF mRNA and the induction of NGF during development.

The present analysis of NGF mRNA expression in a target tissue of NGF-dependent neurons has shown that under appropriate culture conditions epithelial differentiation and NGF mRNA synthesis increase with time in culture. However, only in E11.5 explants the timing of NGF production is comparable to the *in vivo* situation. At earlier stages of development, NGF mRNA expression is low and can be influenced by extrinsic factors. Interestingly, it is strongly stimulated by soluble factors produced by the tissue itself. Identification of this factor may provide clues to the question how the timing of NGF production in different targets is matched to innervation.

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