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# Production of plasminogen activator by migrating cephalic neural crest cells

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**Neural crest cells migrate extensively during embryonic development and differentiate into a wide variety of cell types. Our working hypothesis is that during migration, embryonic cells secrete proteases which modify local microenvironments, thereby facilitating directed cellular movements. In this communication, we report studies on the migration of cephalic neural crest cells in the avian embryo. We demonstrate that these cells produce high levels of the serine protease, plasminogen activator (PA), at the time of their initial migration from the neural tube and during their migration to and colonization of the developing head and neck.**

*Key words:* plasminogen activator/neural crest cells/embryonic development/proteases

## Introduction

During embryonic development, migratory cells encounter tissue components (Wylie *et al.*, 1981), whose degradation may create microenvironmental conditions which affect cellular movements. Such conditions may be established, for example, by the destruction of physical barriers to the penetration of cells (e.g., basement membranes; (Liotta *et al.*, 1979; Smith and Strickland, 1981), or by the partial degradation of molecules, such as fibronectin (Hynes, 1981) or collagen (Kleinman *et al.*, 1981) which are involved in cell adhesion or attachment. The neutral serine protease, plasminogen activator (PA), has properties which suggest that it could be involved in such degradative activities (Strickland, 1980). The enzyme is inducible and thus well suited to provide the regulated, local proteolytic activity that directed cell migration would require. Furthermore, it has been found in a variety of embryonic tissues (Valinsky *et al.*, 1981; Marotti *et al.*, 1982; Bode and Dziadek, 1979), it has been implicated in the colonization and morphogenesis of the bursa of Fabricius by hemopoietic precursors (Valinsky *et al.*, 1981) and its natural substrate, plasminogen, is available to the embryo in abundant amounts throughout development (Valinsky and Reich, 1981).

Neural crest cells migrate extensively during embryonic development and give rise to a wide variety of differentiated cell types. The use of a stable marking technique (Le Douarin, 1969, 1973) in the study of the ontogeny of neural crest derivatives in the avian embryo revealed that the entire skeleton and all of the connective tissue cells of the facial area arise from precursors originating in the cephalic neural crest (Le Lievre, 1974; Le Lievre and Le Douarin, 1975, Le Douarin, 1982). In both chick and quail, cephalic crest migration takes place from embryonic day 2 to day 5. The neural crest cells move underneath the superficial ectoderm to reach the ventral areas of head and neck where cytodifferentiation then occurs. The massive and chronologically well defined migration of cells seemed an appropriate system in which to test the possible involvement of PA in the migratory process. In this article, we report a series of observations made on the crest cells of the mesencephalic and rhomboencephalic regions of the avian embryo. Using a combination of interspecific grafting between quail and chick embryos, PA zymography (Granelli-Piperno and Reich, 1978) and direct enzyme assays (Strickland and Beers, 1976), we show that the production of PA by cephalic neural crest cells is correlated with the onset of their migration from the neural tube and with the period during which they colonize the developing head and neck.

## Results

The migrating neural cell crests at the mesencephalic level were removed from the quail embryo at the 10-somite stage (Figure 1). Grafts of the quail primordium into neural chick embryos were performed at the mesencephalic and rhombencephalic levels (Figure 2).

Assay of PA activity in homogenates of cells from the mesencephalic neural crest revealed an extraordinarily high level of enzymatic activity when compared with that found in adjacent embryonic ectoderm taken from embryos at the same stage or to detergent lysates prepared from cultured quail or chick embryo fibroblasts (Table I). The amount of PA found in mesencephalic neural crest cells was comparable with that found in avian fibroblasts which had been stimulated to produce the enzyme by treatment with phorbol myristate acetate (Wilson and Reich, 1978) or after infection with Rous sarcoma virus (Quigley, 1976).

These findings established that, at the earliest times of migration, mesencephalic crest cells produce PA. A second set of experiments, in which the enzymatic activity of crest cells in the process of colonizing the developing head and neck of quail-chick chimeras, was then performed. Since there are differences in the apparent mol. wts. of chick (40 000 daltons) and quail (43 000 daltons) PAs, the use of chimeras enabled us to ascertain the cellular origin of the enzyme in this region of the embryo. Facial and neck areas of the chimeras were homogenized, the proteins separated by SDS-PAGE and the gels processed by PA zymography. Figure 3 shows that 24 h (lane C) and 48 h (lane D) after the graft was performed, all of the enzyme produced was the 43 000 dalton form, and therefore of donor (i.e., quail) type. We did not observe PA of host (i.e., chick) type, which might have been produced by the superficial ectoderm and/or the pharyngeal endoderm, under these conditions. Standard PAs from phorbol myristate acetate stimulated chick (lane A) or quail (lane B) fibroblasts are shown for comparison. We conclude that the PA in the developing head and neck of the chimeras was produced by migrating cells of neural crest origin.

## Discussion

The results presented in this communication show that PA activity is particularly high in a category of embryonic cells which

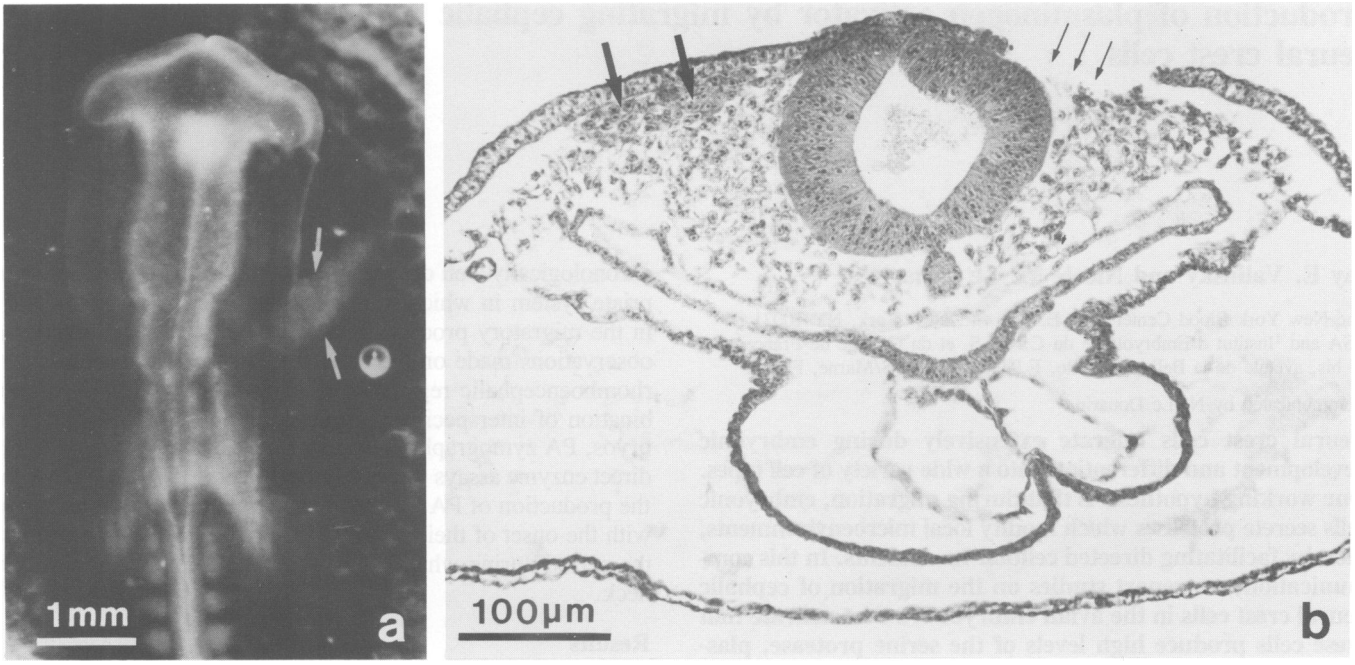


**Fig. 1.** Removal of migrating neural crest cells at the mesencephalic level in a 10 somite quail embryo. **Panel A,** the neural crest cells have been removed on the right side with superficial ectoderm (arrows). **Panel B,** transverse section in the embryo represented in **A**. The migrating mesecephalic neural crest cells can be seen on the left side underneath the ectoderm (arrows). They have been removed on the right (arrows).

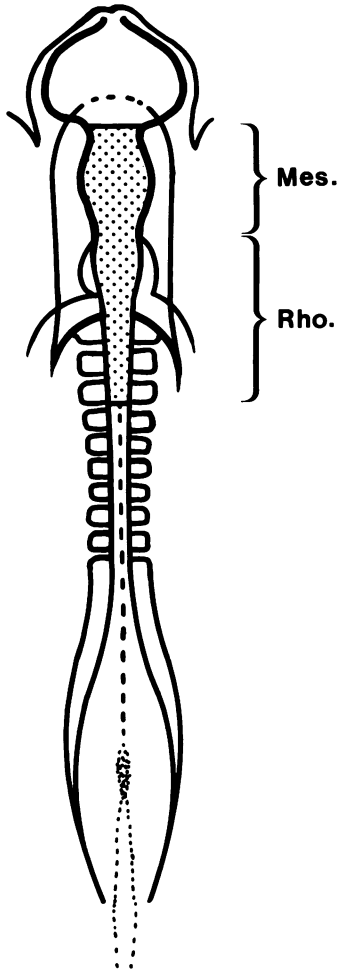


**Fig. 2.** Schematic representation of the levels at which the quail neural primordium is implanted into the chick embryo. Mes, mesencephalon; Rho, anterior rhombencephalon.

**Table I.** PA activity in embryonic avian cells

| Embryonic cell type | PA activity (units urokinase per mg protein) |
|---|---|
| Quail mesencephalic neural crest (migrating) | 336 ± 92 |
| Quail embryo ectoderm (non-migrating) | 25 ± 17 |
| Primary quail embryo fibroblasts (unstimulated) | 52 ± 21 |
| Primary chick embryo fibroblasts (unstimulated) | 40 ± 30 |
| Primary chick embryo fibroblasts (Phorbol ester-treated, 24 h) | 800 ± 130 |
| Primary chick embryo fibroblasts (Rous sarcoma virus-infected) | 500 ± 175 |

Cells were obtained and prepared as described in Materials and methods and assayed for PA activity using the [$^{125}$I]fibrin plate technique. Units of enzyme activity were estimated by comparison of result with urokinase standard curves and specific activities determined on the basis of total protein in the cell preparations.

belong to the neural primordium and which are endowed with remarkable migratory capacities. The neural crest cells which we have studied were removed from the embryo at a time when they were sliding from their initial dorsal position on top of the mesencephalic vesicle to their ventrolateral site of homing to the presumptive facial territory. Three marking systems confirm that the cells used in these experiments were migrating neural crest cells; the localization of migrating crest cells at embryonic days 2 and 3 has been ascertained with quail-chick neural tube chimeras, by the high levels of acetylcholinesterase activity displayed during the migration process (Cochard and Coltey, 1983) and with a monoclonal antibody, $NC_1$, which selectively labels the crest cells at this stage (Vincent and Thiery, 1984). Remarkably similar pictures of crest cell migration have been

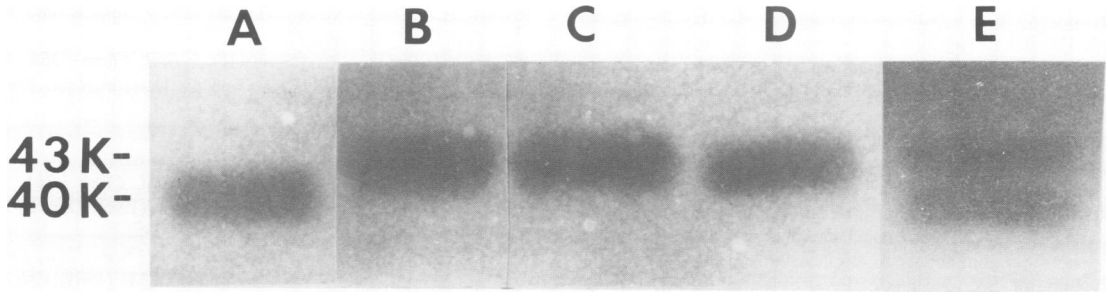


**Fig. 3.** PA zymogram of enzymes present in the facial regions of quail-chick chimeras. The facial regions from quail-chick neural tube chimeras were removed at the indicated times after grafting, homogenized, electrophoresed and processed for assay of PA activity as described in Materials and methods. The figure shows the caseinolytic zones produced in the region of 35 – 50 kd. **Lane A,** conditioned medium from phorbol ester-treated chick embryo fibroblasts; **lane B,** conditioned medium from phorbol ester-treated quail embryo fibroblasts; **lane C,** homogenate of the facial region of a quail-chick chimera 24 h after grafting; **lane D,** 48 h after grafting, **lane E,** mixture of standard quail and chick fibroblast conditioned media.

obtained using these nuclear, cytoplasmic and membrane markers. The adjacent superficial ectoderm, taken at the same developmental stages, as well as normal, cultured fibroblasts, either from quail or chick, had much less PA activity in similar assays.

The analysis of PA production by the cephalic neural crest cells could be taken one step further because of the different electrophoretic mobilities of quail and chick PAs in SDS-polyacrylamide gels. It was known that in quail-chick chimeras in which the mid- and hindbrain vesicles of the quail had been implanted into chick embryos at the appropriate developmental stage, the facial and hypobranchial dermis and skeleton was composed of quail cells, while the pharynx, superficial ectoderm and striated muscles belonged to the chick host (Le Lievre, 1974; Le Lievre and Le Douarin, 1975). Such an experimental model allowed us to investigate whether PA was produced during the modeling of the facial and hypobranchial structures and to determine the cellular source of the enzyme. The overwhelming dominance of the neural crest-derived cells over the resident chick tissue of the face (i.e., pharyngeal endoderm, superficial ectoderm and mesodermally derived tissues) in this process appeared clearly upon electrophoresis. In PA zymographic assays we could not detect PA production by host tissues, while that of the grafted quail tissues was revealed as a conspicuous band of caseinolysis in the indicator gel.

These results, taken together with our previous findings on the colonization of the bursa of Fabricius by the emigrating hemopoietic cells (Valinsky *et al.*, 1981), creates a strong circumstantial case for the involvement of PA in embryonic cell migrations. However, despite the temporal correlations which exist between PA production and cell migration in the embryo, the precise function of the enzyme has not yet been established. A convincing proof that PA is an essential factor in the migration process would come if, for example, it could be shown that antibodies which neutralize PA activity also inhibit migration in a specific way. Antibodies against avian PA are not yet available and those (mono- and polyclonal) which are directed against mammalian enzymes do not cross-react appreciably with either the quail or chick PA, thus making this experiment unfeasible at the present time. In any case, the analogy between the migratory and invasive potentials of some tumor cells (Liotta *et al.*, 1980; Armstrong *et al.*, 1982) and neural crest cells (Erickson *et al.*, 1980) suggests that similar mechanisms might be employed to accomplish what are essentially the same tasks, namely, degradation of extracellular matrix components or basement membranes (Le Douarin, 1982), intra- or extravasation and movement into surrounding tissue (Kramer and Nicholson, 1979; Jones and De Clerck, 1980; Ossowski and Reich, 1983; Liotta *et al.*, 1981). In this respect, fibronectin, one of the major components of the neural crest cell migratory routes (Duband and Thiery, 1982; Thiery *et al.*, 1982) deserves a special mention. As various *in vitro* (Newgreen and Thiery, 1980; Rovasio *et al.*, 1983) and *in vivo* (Boucat *et al.*, 1984a, 1984b) assays have shown, fibronectin plays an essential role in the migratory process. It has been also observed that a certain degree of degradation of this molecule occurs during the migration process both in culture and in the embryo where fibronectin immunoreactivity progressively vanishes while the migration route is used by the crest cells. However, the exact role played by the extracellular matrix component break down and turnover in the migratory process itself is difficult to evaluate quantatively. The fact that PA activity is much higher in the migrating neural crest cells than in other closely apposed tissues remains striking and these findings encourage additional further investigations on the relationship between the migratory potential of embryonic cells and their capacity to produce PA and the microenvironmental (Weston *et al.*, 1978) regulation of the production of PA, or other degradative enzymes (Toole, 1973).

## Materials and methods

*Removal of migrating mesencephalic neural crest cells*

Mesencephalic neural crest cells migrate as a multilayered sheet of cells underneath the superficial ectoderm and can be removed in a virtually pure state by microsurgery in quail or chick embryos at the 8 – 12 somite stage (Smith *et al.*, 1979) (Figure 1). The adjacent ectoderm can also be removed from the ventral side; this material was used as control tissue for PA assays.

*Construction of chimeric embryos*

Isotopic and isochronic grafts of quail neural primordium into 5 – 9 somite chick embryos were performed at the mesencephalic and rhombencephalic levels (Figure 2). The surgical technique used has been described in detail elsewhere (Le Lievre and Le Douarin, 1975). As shown by these authors, cephalic neural crest cells massively invade the facial area during the third and fourth day of incubation. Thus, the facial areas of the chimeras, in which the grafted quail neural crest cells were migrating, were removed at 3.5 and 4.5 days of incubation (i.e., 24 and 48 h after grafting) and processed for PA analysis. Three series of samples were analyzed at each time point.

*Cultures of quail and chick fibroblasts*

Cultures of quail fibroblasts were prepared from 10 day embryos and chick fibroblasts from 11 – 12 day embryos according to published procedures (Quigley, 1976). These primary cultures were passaged once prior to use in PA assays. Chick embryo fibroblasts were treated for 24 h with phorbol myristate acetate (50 ng/ml) under serum-free conditions (Wilson and Reich, 1978) or were infected with a temperature-sensitive mutant Rous sarcoma virus (*ts*-68; the generous gift of Dr. H.Hanafusa, The Rockefeller University, New York, NY) (Quigley, 1976).

*Measurement of PA production by embryonic avian cells*

Mesencephalic neural crest cells, superficial ectodermal cells and cultured

fibroblasts were homogenized in a buffer containing 0.1 M Tris-chloride, pH 8.1, 0.5% Triton X-100 and 100 μg/ml bovine serum albumin (essentially fatty acid free, Sigma Chemical Co.) which had been previously tested for endogenous protease activity. In the case of mesencephalic crest, ~2000–3000 cells could be obtained from each embryo; 20 embryos were used in each of three experiemnts.

PA was assayed using the [$^{125}$I]fibrin plate technique (Strickland and Beers, 1976). Each well of a 24-well plate was coated with a film of [$^{125}$I]fibrinogen (1–1.5 x $10^5$ d.p.m.); incubations were performed at 37°C for 12–24 h in the presence of chicken plasminogen (5 μg/well), which had been prepared by the method of Deutsch and Merz (1970) and treated with diisopropylfluorophosphate to inactivate endogenous plasmin, and Tris-chloride, pH 8.1, BSA (100 μg/ml) buffer in a final volume of 0.3 ml. Units of PA activity, defined in terms of urokinase equivalents, were calculated from urokinase standard curves.

*PA zymography of enzymes derived from grafts of quail mesencephalic neural tube into the chick embryo.*

Chick embryos which had received grafts of the mid- and hindbrain of quail embryos at the same developmental stage (8–12 somites) were sacrificed at day 3.5–4.5. The facial areas and the hypobranchial regions were removed and homogenized in phosphate-buffered saline, pH 7.4 containing 0.5% Triton X-100, 1 μg/ml of soybean trypsin inhibitor, 500 KIU/ml. of Trasylol and 50 mM benzamidine hydrochloride. The samples were analyzed by SDS-PAGE at pH 8.6 (Laemmli, 1970) in 9% polyacrylamide gels. PA activity was reconstituted and assayed in the gels according to a modification of the method of Granelli-Piperno and Reich (1978): the indicator layer contained casein, agar and chicken plasminogen (100 μg/ml). Zones of caseinolysis in the indicator layer, which denote the presence of PA, were visualized by dark field illumination. The apparent mol. wts. of the different species of PA were determined by comparison with mol. wt. standards (Pharmacia Fine Chemicals).

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