Isolation and Characterization of a Factor from Calf Serum That Promotes the Pigmentation of Embryonic and Transformed Melanocytes

JANICE A. JERDAN, HUGH H. VARNER, JUDITH H. GREENBERG, VALERIE J. HORN, and GEORGE R. MARTIN

Laboratory of Developmental Biology and Anomalies, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20205. Dr. Jerdan's present address is Center for Vitreoretinal Research, Wilmer Ophthalmological Institute, Johns Hopkins Medical Institutions, Baltimore, Maryland 21205. Dr. Varner's present address is Cullen Eye Institute, Department of Ophthalmology, Baylor College of Medicine, Houston, Texas 77030. Dr. Greenberg's present address is Genetics Program, National Institute of General Medical Sciences, National Institutes of Health, Bethesda, Maryland 20205.

ABSTRACT A protein (M_r = 63,000) from calf serum that promotes the pigmentation of cultured chick neural crest and mouse melanoma cells has been partially isolated and characterized in this study. The stimulation of melanin synthesis in cultured cells was used to follow its activity during purification. The pigment-promoting factor was isolated by sequential column chromatography on dye-agarose matrices followed by hydroxyapatite and high pressure molecular sieve chromatography. The factor was found to stimulate melanin biosynthesis at 2–4 μg/ml and was specific for melanin-producing cells and their precursors. Antibodies raised in rabbits against the factor inhibited its pigment-promoting activity as well as that of whole calf serum. Enzyme-linked immunoadsorbent assays demonstrated that calf and bovine sera contain molecules that cross-react with the pigment-promoting factor. Horse, human, rat, and chicken sera, which lack the biological activity, also lacked immunological cross-reactivity. Extracts of certain tissues, particularly the submaxillary gland, were observed to be rich sources of pigment-promoting activity.

Neural crest cells appear early in the development of the embryo, migrating out from the neural folds to diverse sites where they differentiate into various phenotypes including fibroblasts, neurons, melanocytes, and chondrocytes (1). It is thought that specific inducers produced by the tissues along these migration routes are important in determining the differentiation of the cells.

Evidence that neural crest cells are pluripotent has been obtained from tissue culture studies. Avian neural crest cells migrate out from cultured neural folds and differentiate into chondrocytes when cultured on a substrate of lysed retinal pigmented epithelium (2) or in the presence of pharyngeal endoderm (3). Neural crest cells in contact with maxillary ectoderm (4) or mandibular epithelium (5) differentiate into osteocytes. Such studies support the concept that neural crest cells can differentiate in specific ways in response to factors associated with various tisuses.

In the absence of such exogenous inducers, neural crest cells differentiate in vitro into melanocytes under standard culture conditions (fetal calf serum, Eagle's medium) (6). This is a rather surprising result since only a very minor portion of the cranial neural crest differentiates into melanocytes in vivo (1). More recently, it was observed that in medium supplemented with horse seurm, neural crest cells assume characteristics of cholinergic neurons (7). These studies suggest that horse serum and calf serum contain different factors which are able to induce distinct phenotypes.

Here we report the partial isolation of a factor from calf serum that promotes melanogenesis. We call this activity pigment-promoting factor (PPF).[1] This factor is a protein

[1] *Abbreviations used in this paper:* CS, calf serum; DME, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; PPF, pigment-promoting factor; TCA, trichloroacetic acid.

which promotes the differentiation of neural crest cells into melanin-producing cells and the synthesis of melanin by the C3 clone of B16 murine melanoma cells (8) in a dose-dependent manner. The B16 melanoma cells had previously been found to require fetal calf serum for melanogenesis (9–11).

MATERIALS AND METHODS

Neural Crest Cell Cultures: Neural crest cells were isolated and cultured as described by Greenberg and Schrier (7). In brief, neural tubes with associated neural crest and ectoderm were explanted from chick embryos (Hamburger and Hamilton stage 8–9) (12) and placed in 35-mm tissue culture dishes containing Dulbecco's modified Eagle's medium (DME)[1] and Ham's F12 (1:1) with 5% calf serum (CS) and 1% chick embryo extract. After 48 h, the neural crest cells had migrated away from the explant. At that time, the neural tubes and the ectoderm were removed mechanically, and the neural crest cells were detached from the dishes with trypsin and replated as dense spots of cells (6,000–10,000 cells/15 μl) (13). The cells were allowed to attach for 90 min, after which the dishes were flooded with 2 ml of medium. Cells were assayed for melanin synthesis 3 d later.

Mouse Melanoma Cells: B16/C3 cells, obtained from Drs. Dean Engelhardt (New York University), Paul Fisher (Columbia University), and John Kreider (Hershey Medical Center) were maintained in DME with 5% CS and 2 mM L-tyrosine. 2 d before being assayed for melanin biosynthesis, the cells were plated at a density of 60,000 per 35-mm dish.

Assay for Melanin Biosynthesis: Melanin biosynthesis was assayed in both neural crest and B16/C3 cells as described by Greenberg and Oliver (13). Subcultured neural crest cells were maintained in 2 ml of a mixture of DME and Ham's F12 media (1:1) containing 1% CS, 1% chick embryo extract, and varying amounts of the material to be tested for pigment-promoting activity. These low levels of serum and embryo extract were necessary to maintain cell viability. To induce pigmentation, one group of six culture dishes received medium containing an additional 4% CS (positive control), while the other groups of six dishes received medium with 1% CS and various additives to be tested for activity. After 72 h, 0.5 μCi [^{14}C]tyrosine was added to each of four dishes in every group, two with and two without phenylthiourea (0.24 mM), a specific inhibitor of tyrosinase (14). After a 5-h labeling period, the neural crest cells were detached from the dishes with 0.25% trypsin and suspended in cold 20% trichloroacetic acid (TCA). Precipitated material was isolated by centrifugation, resuspended and washed twice with cold 10% TCA, then solubilized in NCS (New England Nuclear, Boston, MA), and its radioactivity was determined by liquid scintillation counting. Cells in the remaining pair of dishes in each group were removed with 0.25% trypsin and 0.1% EDTA and counted in an electronic particle counter (Coulter Electronics, Hialeah, FL). The B16/C3 melanoma cells were subcultured at 60,000 cells per 35-mm dish and were assayed using the same protocol after 48 h.

One set of melanin biosynthesis assays using the B16/C3 cells involved an adaptation of the method of Mufson et al. (9). In this study, 35-mm dishes of cells plated at 60,000 cells per dish were grown in DME/Ham's F12 (1:1) containing 5% CS. When the cultured cells were almost confluent, the plates were rinsed twice in balanced salt solution and flooded with a defined serum-free medium for melanoma cells (15). Materials to be tested for stimulatory activity were then added to the dishes in triplicate. Defined medium containing 5% CS was used as a positive control for stimulation. Fresh medium was substituted 2 and 4 d later, and medium from all dishes was harvested 48 h after that. Following acidification with 5 μl of 10 N HCl per sample, the absorbance at 600 nm was determined. This modification of the method of Mufson et al. (9) was made in order to eliminate the interference of phenol red present in the medium. This assay proved to be simpler and equally accurate in determining melanin biosynthesis in the B16/C3 line.

Purification of PPF: In all cases, fractions to be assayed were dialyzed against DME and assayed for PPF activity. A 40–60% saturated $(NH_4)_2SO_4$ fraction was prepared from calf seurm (Pel-Freez Biologicals, Rogers, AR), and the precipitate was resuspended in PBS. After extensive dialysis against 50 mM Tris-HCl (pH7.4) plus 25 mM 6-aminohexanoic acid, the serum fraction was chromatographed on a column (2.5 × 35 cm) of Blue Agarose (Bethesda Research Laboratories, Gaithersburg, MD) in the same buffer. The unbound portion contained PPF, which was then chromatographed on a Matrex Gel Blue B column (2.5 × 15 cm; Amicon Corp., Danvers, MA) in the same buffer. PPF bound to the Blue B resin and was eluted from the column with 2 M NaCl. Following dialysis against 1 mM K_2HPO_4 (pH 6.8), the material that contained the active fraction was passed over a column (1 × 10 cm) of hydroxyapatite (HA-Ultrogel, LKB Instruments, Inc., Gaithersburg, MD) and eluted with a linear gradient of sodium phosphate (1–500 mM, pH 6.8). A single peak of activity eluted between 250 and 350 mM phosphate. This active material was concentrated by precipitation with 65% saturated $(NH_4)_2SO_4$, dissolved and dialyzed against 8 M urea in 10 mM Tris-HCl (pH 7.4), and then applied to a molecular sieve column (TSK 2000, Beckman Instruments, Inc. Fullerton, CA) using a Beckman model 332 high pressure liquid chromatograph. Fractions (0.5 ml) were eluted at 0.5 ml/min.

Gel Electrophoresis: Relative molecular weights were determined by SDS PAGE essentially as described by Laemmli (16).

Preparation of Antibodies to PPF: Purified PPF (300 μg) in complete Freund's adjuvant was injected subcutaneously into rabbits. 2 wk later, the rabbits were reinjected with PPF (150 μg) in incomplete Freund's adjuvant. 3 wk later, blood was drawn and the IgG component of the serum was separated from other proteins by chromatography on a DEAE-Affigel Blue column (1 × 8 cm) (Bio-Rad Laboratories, Richmond, CA) in 0.02 M KH_2PO_4, pH 8.0, according to the procedure described by the manufacturer.

Enzyme-linked Immunoadsorbent Assays (ELISA): ELISA assays were conducted as described by Rennard et al. (17). Varying amounts of PPF and other factors in Voller's buffer (0.1 M sodium carbonate and 0.02% sodium azide, pH 9.6) were adsorbed overnight to the surface of microtiter wells (Costar Data Packaging, Cambridge, MA). For direct analyses, rabbit antiserum against PPF was added to the wells for 1 h and then the wells were rinsed with PBS-Tween (0.05%). Goat anti-rabbit IgG conjugated to horseradish peroxidase (Cappel Laboratories, Cochranville, PA) was added to the wells, and bound enzyme was detected with *o*-phenylenediamine by absorbance at 492 nm.

RESULTS

Addition of calf serum caused a dose-dependent increase in melanin synthesis by chick neural crest cells and by mouse B16/C3 melanoma cells which plateaued at higher levels (Fig. 1). Neural crest and B16/C3 cells showed very similar responses in the melanin biosynthesis assay to increasing amounts of serum (Fig. 1) and to the material at various stages of purification. When horse serum was substituted for either calf or fetal calf serum, little or no synthesis of melanin was observed by either the neural crest (not shown) or the B16/C3 cells (Fig. 1). The incorporation of labeled tyrosine into melanin was used as a measure of melanogenesis in the purification of PPF from serum.

The factor promoting melanogenesis has been partially isolated from calf serum. Greater than 60% of the PPF activity in calf serum was precipitated at 40–60% $(NH_4)_2SO_4$ (Table I). PPF activity did not bind in significant amounts to Blue Agarose (Fig. 2*A*) but did bind to Matrex Blue B (Fig. 2*B*) and to hydroxyapatite (Fig. 2*C*). A considerable purification

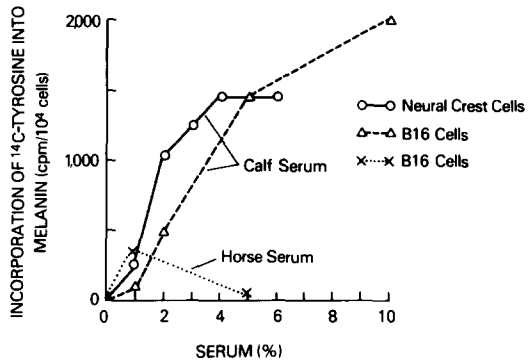

FIGURE 1 Dependence of pigmentation on calf serum concentration. [^{14}C]Tyrosine incorporation into melanin was determined in spot cultures of 8–10 × 10^3 neural crest cells after 72 h in culture. B16/C3 cells were plated at 6 × 10^5 cells per 35-mm dish, and the incorporation of label was determined at 48 h as described in Materials and Methods. Each point is the average of six individual cultures.

was achieved by these steps (Table I). PPF activity was further purified by high performance liquid chromatograhy gel filtration (Fig. 2 *D*). SDS PAGE of material from the active peak showed a major Coomassie Blue-staining band migrating at $\sim M_r = 63{,}000$ (Fig. 3). The final step in purification resulted in over a 2,400-fold enrichment for the factor from the original serum (Table I). Melanin synthesis was measured in melanoma cells cultured with serum, with PPF from the 40–60% saturated $(NH_4)_2SO_4$ precipitation and with more highly purified PPF (Fig. 4). Half maximal melanogenesis was achieved with 2 μg/ml of the PPF preparation.

The sensitivity of PPF to various enzymes (Table II) was used to determine the chemical nature of PPF. A considerable part of the activity was lost after incubation with trypsin whereas deoxyribonuclease and ribonuclease treatments were without effect. PPF was active after exposure to 8 M urea but was inactivated by exposure to 4 M guanidine as well as to elevated temperature (Table II). PPF was stable after storage at −70°C for 6 mo (not shown). These observations suggest that PPF is a protein.

Rabbits were immunized with PPF. Subsequently, high-titer antibodies were detected, and an IgG fraction was prepared by chromatography of the serum on a DEAE-Affigel Blue column (Bio-Rad Laboratories). This partially purified IgG fraction, when added to the culture medium of B16/C3 cells, was found to inhibit pigmentation of the cells in the presence of either added PPF or calf serum (Fig. 5) while the same fraction from preimmune serum had no such effect. Further, the inhibition of pigmentation was specific since the anti-PPF antibody did not alter the rates of protein synthesis or cell division (data not shown). A comparable IgG fraction from preimmune rabbit serum had no effect on PPF activity (Fig. 5). These studies indicate that PPF is the major factor in calf serum promoting pigmentation.

The anti-PPF antiserum was used to quantitate PPF in various sera and tissue extracts. These studies showed that fetal calf, calf, and bovine sera contained similar levels of PPF by ELISA while human, horse, chicken, pig, and rat sera showed no detectable cross-reaction with anti-PPF antibodies under the conditions used (Table III). Extracts of a variety of bovine tissues exhibited cross-reactivity with anti-PPF antibodies (data not shown) and were able to induce pigmentation

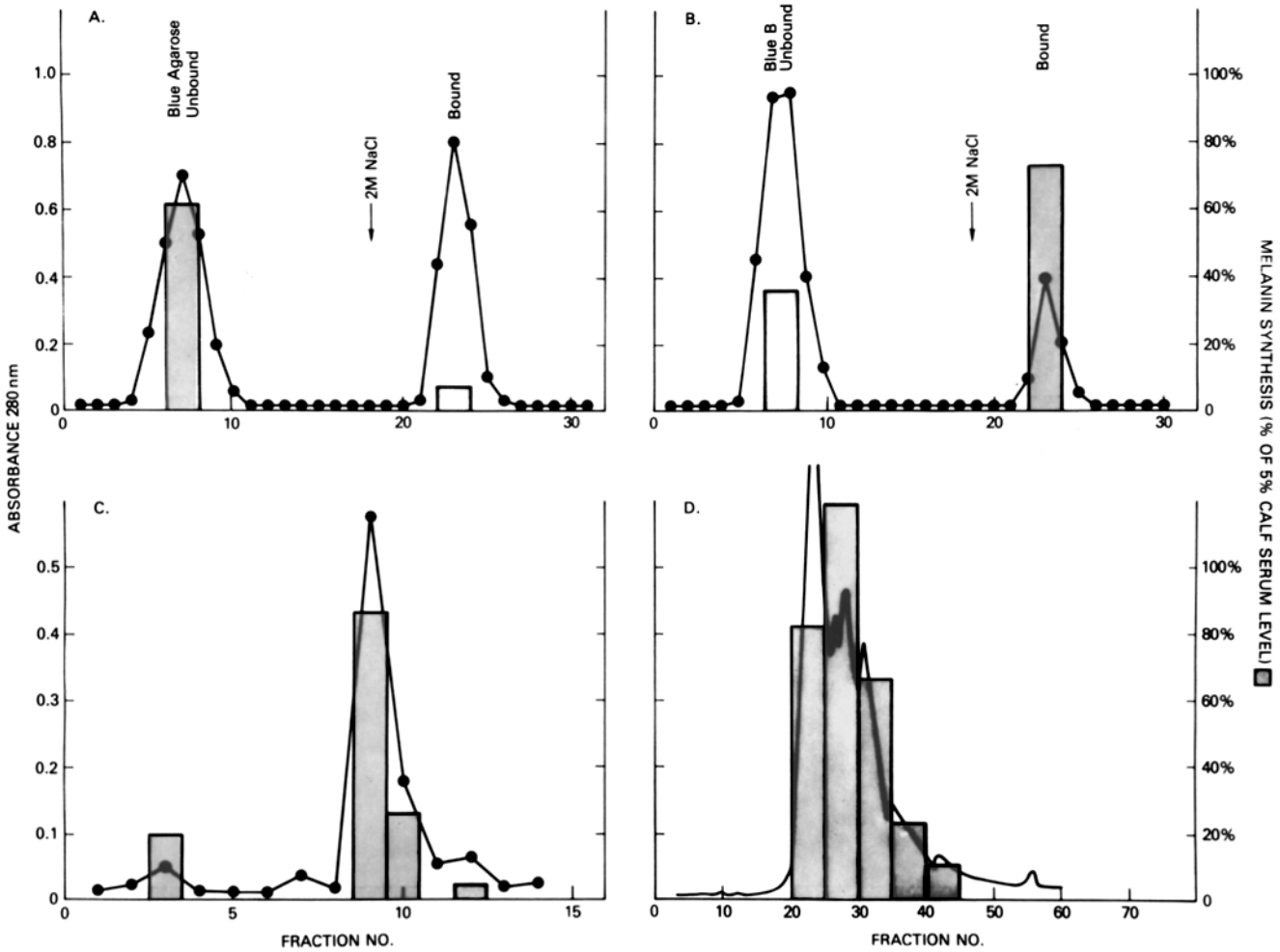

FIGURE 2 Purification of PPF. In each case, the protein elution profiles are plotted against the ability to stimulate melanin synthesis in cultures of B16/C3 mouse melanoma cells. Material eluted from Blue Agarose (*A*) and Mātrex Gel Blue B (*B*) columns with column buffer (unbound fraction) followed by 2 M NaCl (bound fraction). (*C*) Elution profile of bound fraction from Mātrex Gel Blue B during separation on an hydroxyapatite column in an increasing sodium phosphate gradient. (*D*) Molecular sieve chromatography in 8 M urea of active material from the hydroxyapatite separation of PPF. For this latter column the following molecular weight markers were used; ovalbumin (45,000), BSA (66,000), and ribonuclease (13,363). The active material eluted between ovalbumin and BSA.

TABLE I

Isolation and Recovery of Pigment-promoting Activity from Calf Serum

	Total protein	Specific activity*	Recovery of activity	Fold purification
	mg		*%*	
Calf serum	84,000	27	100	1
40–60% Ammonium sulfate precipitate	6,730	119	111	4
Blue Agarose – unbound fraction	4,320	242	75	9
Mātrex Blue B – bound fraction	43	3,900	38	144
Hydroxyapatite – active peak	3	13,000	6	482
Molecular sieve – active peak	1	65,000	3	2,407

Protein concentrations were determined from the absorbance values at 260 and 280 nm (18). Material was tested for its pigment-promoting activity in neural crest cultures in the presence of 1% CS and 1% embryo extract. The level of incorporation of [^{14}C]tyrosine specifically into melanin was defined as the difference in levels of [^{14}C]tyrosine incorporation into TCA-insoluble material in the presence and absence of phenylthiourea, a tyrosinase inhibitor.

* Specific activity = TCA – precipitable melanin-specific cpm × 10^{-4} cells/milligrams of protein.

in cultures of B16/C3 cells with the highest level of activity observed in extracts of submaxillary gland (Table IV).

DISCUSSION

Chick neural crest cells differentiate into melanocytes when cultured in the presence of calf serum. Since neural crest cells are pluripotent and few form melanocytes in vivo, it appeared that a factor or factors in calf serum directs their development. Here we report the partial isolation and characterization of a factor in calf serum that induces the differentiation of neural crest cells into melanocytes. A significant enrichment of the factor was achieved by chromatographic procedures. On the basis of its apparent size ($M_r \simeq 63{,}000$) and susceptibility to trypsin and heat, it would appear to be a protein which we have termed pigment-promoting factor (PPF).

Using a quantitative assay for melanin synthesis, we found that calf serum also stimulated the pigmentation of mouse B16/C3 melanoma cells and that highly purified PPF showed this activity. Previous studies showed the pigmentation of melanocytes was also dependent on serum factors (9, 10). Furthermore, using antibodies against purified PPF, we found that we could prevent neural crest cells and B16/C3 cells from pigmenting in culture in the presence of calf serum. These studies indicate that PPF is the major pigmenting factor in serum.

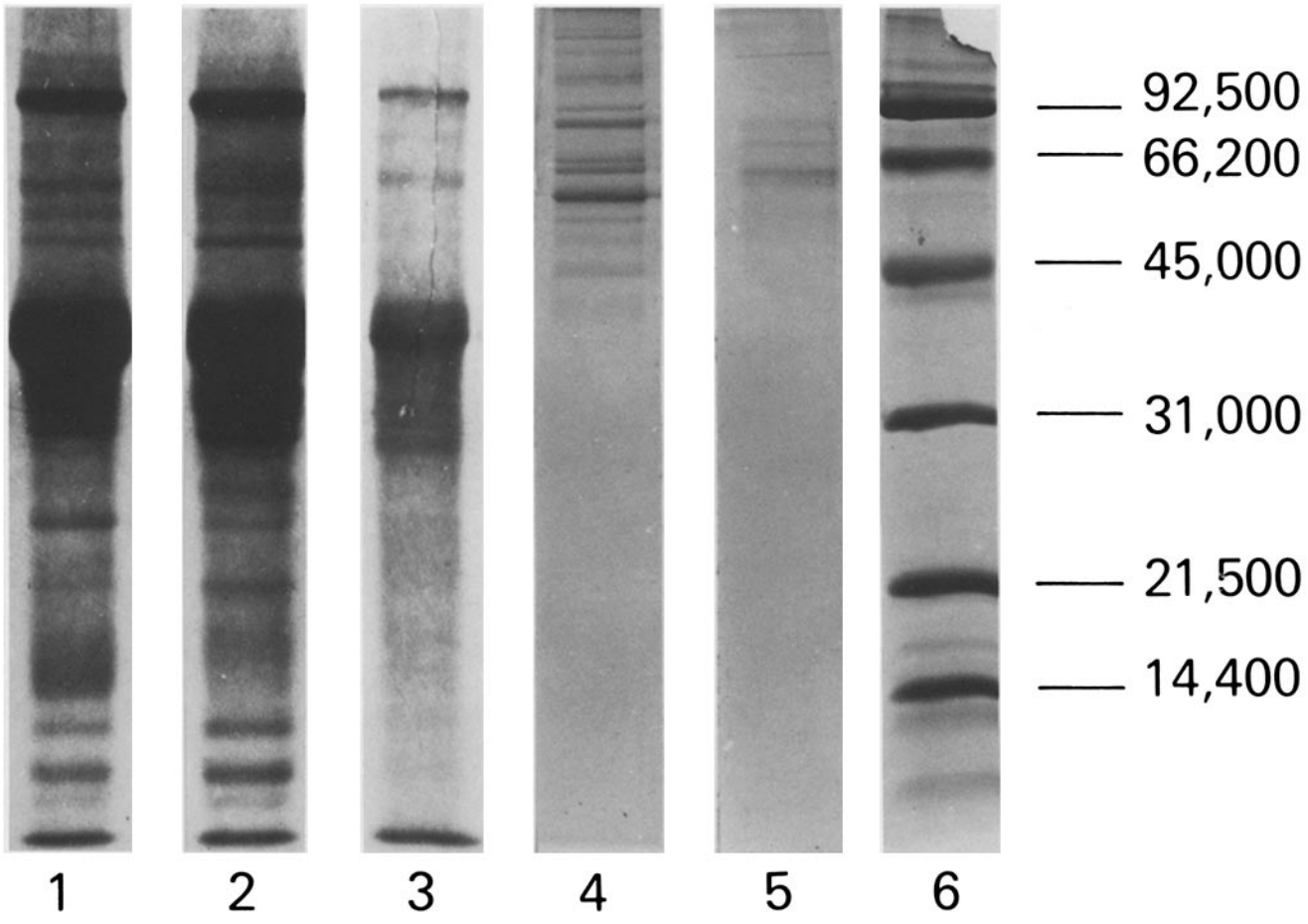

FIGURE 3 Electrophoretic separation of active material. Following reduction in dithiothreitol, proteins were separated on a 7.5% polyacrylamide gel in SDS and stained with Coomassie Blue. (Lane 1) 40–60% saturated $(NH_4)_2SO_4$ precipitate, (lane 2) Blue Agarose unbound fraction, (lane 3) Blue B bound fraction, (lane 4) active peak from hydroxyapatite, (lane 5) active peak from molecular sieve, (lane 6) molecular weight standards.

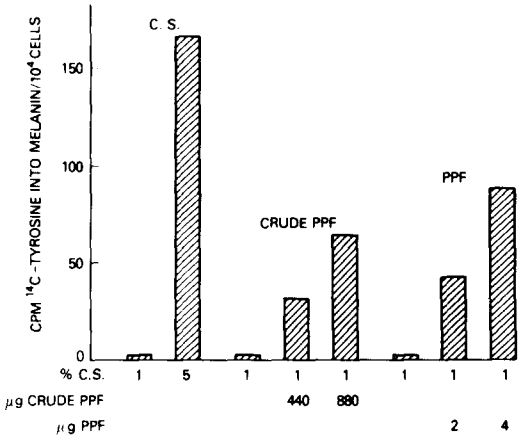

FIGURE 4 Effect of calf serum and PPF on cultured embryonic chick neural crest cells. Whole serum, PPF from the 40–60% saturated $(NH_4)_2SO_4$ precipitate, and highly purified PPF from the hydroxyapatite active peak were used. Determinations of melanin synthesis were made as described in Materials and Methods.

TABLE II

Activity of PPF after Various Treatments

Culture conditions	PPF treatment	Activity
		%
5% CS		100
1% CS		25
1% CS + PPF (3 μg/ml)	70°C, 30 min	59
1% CS + PPF (3 μg/ml)	37°C, 18 h	90
1% CS + PPF (3 μg/ml)	37°C, Trypsin	23
1% CS + PPF (3 μg/ml)	37°C, RNase	105
1% CS + PPF (3 μg/ml)	37°C, DNase	119
1% CS + PPF (3 μg/ml)	4°C, 8 M Urea	82
1% CS + PPF (3 μg/ml)	4°C, 4 M Guanidine-HCl	12

Trypsin digestion was carried out using trypsin bound to CNBr-activated Sepharose 4B, and the trypsin was removed from the PPF after digestion by filtration. Digestion was carried out in the presence of 100 μg PPF and 1.5 ml beads. 75 μg of PPF was incubated with 25 μg/ml of RNase or DNase for 1 h in Earle's balanced salt solution. Treatment with 8 M urea or 4 M guanidine-HCl was carried out by extensive dialysis of PPF against either urea or guanidine-HCl in PBS. Activity is expressed as the percent of incorporation of [^{14}C]tyrosine into melanin in B16/C3 melanoma cells in the presence of 5% calf serum. Control groups were incubated under identical conditions without trypsin, or with RNase or DNAse in balanced salt solution.

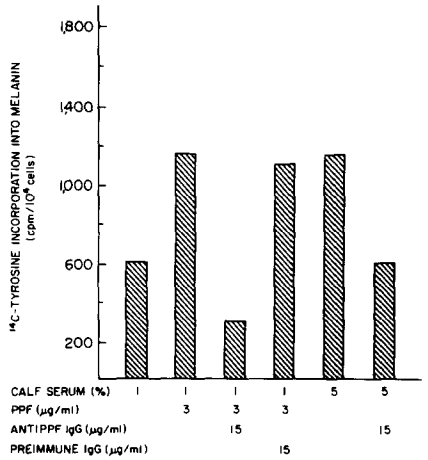

FIGURE 5 Effect of anti-PPF IgG on the pigment-promoting activity of PPF and whole calf serum. The IgG fraction against PPF was tested for its effect on melanin synthesis by B16/C3 cells in the presence and absence of PPF. A comparable IgG fraction from preimmune rabbit serum was also used as a control.

TABLE III

Presence of Immunoreactive PPF in Various Sera and Embryo Extract

Serum	Immunoreactive PPF*
	%
Chicken	0
Horse	0
Human	0
Rat	0
Fetal calf	80
Calf	92
Bovine	93
Chicken embryo extract	0

An indirect (inhibition) ELISA was performed in triplicate to measure the ability of various sera and embryo extract to block the binding of anti-PPF to PPF. PPF was coated onto microtiter plates in Voller's buffer at a concentration of 1 μg per well. Sera were diluted (1:2028) in PBS/Tween and allowed to react overnight at 4°C in suspension with antibodies against PPF at a constant dilution (1:320). The mixtures were then transferred to the antigen-coated wells and allowed to react as described in Rennard et al. (17). Sera that blocked the binding of anti-PPF to PPF-coated wells contained material that cross-reacted with PPF.

* Values are expressed as percentage inhibition of ELISA.

TABLE IV

Tissue Distribution of Pigmentation Activity

Addition	Melanogenesis
	$A_{600}/10^6$ cells
None	0.1
PPF	4.3
Calf serum	2.8
Submaxillary gland extract	2.7
Adrenal gland extract	1.0
Pituitary gland extract	0.9
Ovary extract	1.0

PPF (3 μg/ml), calf serum (5%), or extracts (100 μg/ml) were added to cultures of B16/C3 melanoma cells, and the accumulation of melanin in the medium was quantitated from day 4 to day 6 as $A_{600}/10^6$ cells. Tissues were weighed, ground in a meat grinder and added to a volume of 4 M guanidine-HCl in PBS equal to the starting tissue weight. The extractions were carried out overnight and the extracts were dialyzed against medium before being tested.

Various observations indicate that PPF is distinct from α-melanocyte-stimulating hormone. Whereas the latter promotes the pigmentation of B16/C3 melanoma cells, it does not induce neural crest cells to pigment when added to the cells in medium containing 1% calf serum and embryo extract (data not shown). Moreover, the antibodies prepared against PPF showed no reaction in ELISA tests with α-melanocyte-stimulating hormone (unpublished data).

Various fluids and tissue extracts were assayed for PPF by immunoassay and by their ability to promote pigmentation of neural crest and melanoma cells in culture. These studies showed a close correlation between the two assays. Chicken, rat, human, and horse sera lacked pigment-promoting activity and immunoreactive material. Extracts of certain tissues including submaxillary gland, pituitary gland, and ovary showed pigment-promoting activity and immunoreactive material. Extracts of the submaxillary gland showed considerably more activity than any other tissue or nonbovine serum tested. However, known factors in the submaxillary gland including nerve growth factor and epidermal growth factor lacked this activity (data not shown). Further, PPF did not alter the differentiation of various other cells including chick myoblasts, limb bud cells, or chondrocytes. These studies indicate that PPF shows a distinct distribution and appears to be specific for inducing pigmentation.

Evidence for a specific molecule in calf serum capable of promoting the phenotypic differentiation of both embryonic and transformed cells is particularly interesting in view of a report by Laskin et al. (10) which suggested that distinct factors in fetal calf serum stimulate the synthesis and secretion of melanin in B16/C3 cells. The effect of PPF on melanin secretion is not known, but it is clear that the serum factor described here stimulates melanin synthesis. Another pigment-promoting factor has been described recently. Derby and Newgreen (19) reported that embryonic quail trunk neural crest cells differentiate into melanocytes in the presence of chick embryo extract or fetal calf serum or when associated with the original neural tube. The authors suggested that the factor responsible for enhancing pigmentation is a large molecule ($>M_r$ = 400,000) present in chick embryo extract but only in rare lots of fetal calf serum. Their report contrasts with our findings in two important ways: their estimated molecular weight is much higher, and PPF is found in all calf and bovine sera tested. For these reasons, it is likely that the pigmentation-promoting factor described by those authors is distinct from that reported in this paper.

We report here the isolation of a molecule that induces a differentiated phenotype only in pigmenting cells and their precursors. Although the factor responsible for inducing chick melanocyte differentiation in vivo may not be identical to the circulating serum protein described here, the end result is the same. The protein described in this study may be useful in studying the process of melanocyte differentiation.

We thank Drs. Hynda Kleinman and Gary Grotendorst for their helpful comments on the manuscript and Ms. Irma Burke for typing the manuscript.

This work was supported in part by National Institutes of Health fellowships, 5-F32HD05812 and 5-F32DE05336, to J. A. Jerdan.

Received for publication 31 May 1984, and in revised form 25 January 1985.

REFERENCES

1. Weston, J. A. 1971. The migration and differentiation of neural crest cells. *Adv. Morphog.* 8:41–114.
2. Newsome, D. A. 1976. In vitro stimulation of cartilage in embryonic chick neural crest cells by products of retinal pigmented epithelium. *Dev. Biol.* 49:496–507.
3. Holtfreter, J. 1968. Mesenchyme and epithelia in inductive and morphogenetic processes. *In* Epithelial-Mesenchymal Interactions. R. Fleischmajer and R. E. Billingham, editors. Williams and Wilkins, Baltimore. 1–30.
4. Bee, J., and P. Thorogood. 1980. The role of tissue interactions in the skeletogenic differentiation of avian neural crest cells. *Dev. Biol.* 78:47–62.
5. Hall, B. K. 1980. Tissue interactions and the initiation of osteogenesis and chondrogenesis in the neural crest-derived mandibular skeleton of the embryonic mouse as seen in isolated murine tissues and in recombinations of murine and avian tissues. *J. Embryol. Exp. Morphol.* 58:251–264.
6. Dorris, F. 1938. The production of pigment in vitro by chick neural crest. *Roux Arch.* 138:323–334.
7. Greenberg, J. H., and B. K. Schrier. 1977. Development of choline acetyltransferase activity in chick cranial neural crest cells in culture. *Dev. Biol.* 61:86–93.
8. Kreider, J. W., and M. E. Schmoyer. 1975. Spontaneous maturation and differentiation of B16 melanoma cells in culture. *J. Natl. Cancer Inst.* 55:641–647.
9. Mufson, R. A., P. B. Fisher, and I. B. Weinstein. 1979. Effect of phorbol ester tumor promoters on the expression of melanogenesis in B16 melanoma cells. *Cancer Res.* 39:3915–3919.
10. Laskin, J. D., R. A. Mufson, I. B. Weinstein, and D. L. Engelhardt. 1980. Identification of a distinct phase during melanogenesis that is sensitive to extracellular pH and ionic strength. *J. Cell. Physiol.* 103:467–474.
11. Laskin, J. D., L. Piccinini, D. L. Englehardt, and I. B. Weinstein. 1982. Control of melanin synthesis and secretion by B16/C3 melanoma cells. *J. Cell. Physiol.* 113:481–486.
12. Hamburger, V., and H. L. Hamilton. 1951. A series of normal stages in the development of the chick embryo. *J. Morphol.* 88:49–92.
13. Greenberg, J. H., and C. Oliver. 1980. Dimethyl sulfoxide reversibly inhibits the pigmentation of cultured neural crest cells. *Arch. Biochem. Biophys.* 204:1–9.
14. Whittaker, J. R. 1971. Biosynthesis of a thiouracil pheomelanin in embryonic pigment cells epxosed to thiouracil. *J. Biol. Chem.* 246:6217–6226.
15. Sato, G., and L. Reid. 1978. Replacement of serum in cell culture by hormones. *Int. Rev. Biochem.* 20:219–251.
16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
17. Rennard, S. I., R. Berg, G. R. Martin, J. M. Foidart, and P. Gehron Robey. 1980. Enzyme-linked immunoassay (ELISA) for connective tissue components. *Anal. Biochem.* 104:205–214.
18. Kalckar, H. M. 1947. Differential spectrophotometry of purine compounds by means of specific enzymes. III. Studies of the enzymes of purine metabolism. *J. Biol. Chem.* 167:461–475.
19. Derby, M. A., and D. F. Newgreen. 1982. Differentiation of avian neural crest cells in vitro: absence of a developmental bias toward melanogenesis. *Cell Tissue Res.* 225:365–378.