Control of Tyrosinase Gene Expression and Its Relationship to Neural Crest Induction in Rana pipiens

II. MEASUREMENT OF TYROSINASE mRNA ACCUMULATION DURING EARLY EMBRYOGENESIS USING A SPECIFIC cDNA PROBE

Glen N. Gaulton† and Edward L. Triplett
From the Department of Biological Sciences, University of California, Santa Barbara, California 93106

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The relationship between the process of neural induction and the control of tyrosinase gene expression in the cells that derive from the neural crest of amphibians has been examined at the molecular level. [3H]tyrosinase cDNA was utilized as a probe to measure the levels of tyrosinase RNA transcripts present in Rana pipiens embryos from the time of fertilization through Stage 25 of cleavage (operculum complete, 240 h) and to correlate these levels with those previously published for the rate of tyrosinase protein synthesis.

R. pipiens [3H]tyrosinase cDNA was synthesized from a purified tyrosinase mRNA template using avian myeloblastosis virus reverse transcriptase and was enriched for full length copies by preparative polyacrylamide gel electrophoresis. This cDNA product was estimated to represent >90% by length of tyrosinase mRNA and hybridized to tyrosinase mRNA to >97% within two orders of magnitude of Rot values. The extent of hybridization of [3H]tyrosinase cDNA to total embryonic RNA throughout early development paralleled the rate of synthesis of tyrosinase protein. Tyrosinase RNA transcripts were first detected at Stage 12-13 (0.0032% of total RNA) and rose to maximal levels by Stage 19 (0.011%). This represents a 50-fold increase from preinduction levels. These results are consistent with a model which predicts that one of the early events in the development of neural crest derivatives is the transcriptionally dependent accumulation of functionally mature tyrosinase mRNA.

The enzyme tyrosinase (EC 1.10.3.1, o-diphenol:O2 oxidoreductase) is a particularly useful marker for studying the differentiation of neural crest derivatives in amphibians. The synthesis of tyrosinase is unique to neural crest derivatives, as determined by both morphological (1-4) and biochemical criteria (5, 6). Synthesis of tyrosinase zymogen is first detectable in neural plate embryos (Stage 13), immediately following determination of the neural crest at midlate gastrula (Stage 11-12) (7). Stage 13 also marks the onset of nascent, polysome-bound tyrosinase activity (8). Benson and Triplett (8) have shown that treatment of midastrula embryos with actinomycin D, at levels which strongly inhibit uridine incorporation into nucleic acids but not protein synthesis, inhibits the induction and accumulation of tyrosinase enzyme by 75-100%, thus suggesting that expression of the tyrosinase gene is a transcriptionally dependent event. The molecular details of the relationship between neural induction and the accumulation of functionally mature tyrosinase mRNA remain undefined.

In the accompanying paper (9), we have described the isolation of full length Rana pipiens tyrosinase mRNA, which was judged by both physical and functional criteria to be >98% pure. In this paper, we describe the use of purified tyrosinase mRNA as a template for the synthesis of full length [3H]tyrosinase cDNA and the use of this [3H]cDNA as a probe to quantify the accumulation of tyrosinase RNA transcripts during R. pipiens embryogenesis. A comparison of these values with those published previously for the rate of tyrosinase protein synthesis throughout early embryogenesis (7) indicates that both the level of tyrosinase mRNA transcripts and the rate of tyrosinase protein synthesis increase dramatically at the conclusion of embryonic induction coincident with morphological determination of the neural crest.

EXPERIMENTAL PROCEDURES AND RESULTS

Measurement of the Accumulation of Tyrosinase mRNA during R. pipiens Early Embryogenesis—To accurately detect tyrosinase RNA transcripts present in Stage 1-Stage 25 embryos, a titration technique was employed in which a fixed amount of [3H]tyrosinase cDNA (1.0 ng, approximately 2000 cpm) was annealed at 43 °C with increasing amounts of total cellular RNA under stringent hybridization conditions. The theoretical considerations of this approach and the treatment of results have previously been described (17). This methodology has proven to be particularly well suited to analysis of the levels of specific RNA transcripts in developing systems where wide fluctuations in both the number of specific transcripts and total RNA mass are often seen (18, 24-29).

The applicability of this titration method to these studies

The abbreviations used are: AMV, avian myeloblastosis virus; mlL-1, mole second liter-1; EtBr, ethidium bromide; Rot, initial concentration of RNA (in moles of nucleotide/liter) times the duration of the hybridization reaction (in seconds); Dst, initial concentration of DNA times; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; DTT, dithiothreitol.

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† To whom correspondence should be addressed. Present address, Department of Cancer Biology, Harvard University School of Public Health, 665 Huntington Ave., Boston, MA 02115.
is demonstrated in the hybridization of tyrosinase cDNA to increasing amounts of tyrosinase mRNA (Fig. 4A). Maximal levels of hybridization for tyrosinase mRNA were obtained at input RNA/cDNA ratios of 1.2-1.4 which indicates that at least 85% of the tyrosinase mRNA sequence is represented in tyrosinase cDNA. Theoretically, saturation of mRNA with full length cDNA should be achieved at a 1:1 RNA/cDNA ratio. However, in practice, complete saturation at ratios lower than 1.5 have not been observed (13, 17, 18, 27).

To determine the time course of appearance of tyrosinase gene transcripts, [3H]tyrosinase cDNA was hybridized to a D_{0.2} of 0.22 mol s liter{\textsuperscript{-1}} with increasing amounts of total cellular embryonic RNA isolated from successive embryonic stages. As demonstrated in Fig. 4, B and C, no tyrosinase gene transcripts were detected in embryonic RNA prepared from Stages 1 (unfertilized egg, 0 h), 3-4 (early cleavage, 4 h), 7-8 (blastula, 12 h), and 10-11 (crescent gastrula, 25 h). The high specific activity of our tyrosinase cDNA (1.97 × 10{\textsuperscript{5}} cpm/μg) coupled with the ease of isolating large amounts of R. pipiens embryonic RNA enabled a maximal RNA/cDNA ratio of 30,000 in these studies. Assuming the reliable detection of 5% hybridization at a RNA/DNA ratio of 30,000 (this allows for nonspecific fluctuations of two times background), a minimal 14-fold amplification over preinduction levels. Increasing levels of tyrosinase RNA were first detectable at Stage 14-15 (neurula) (0.0057%) through Stage 19 (early tailbud) (0.0093%) and Stage 25 (feeding) (0.0111%), representing a 50-fold amplification overall. Following Stage 19, the levels of tyrosinase mRNA declined slightly but steadily through Stage 21 (mid-tailbud) (0.0093%) and Stage 25 (feeding) (0.0088%) (Fig. 4E).

Throughout the period of induction and amplification of tyrosinase RNA transcripts, the concentration of total RNA per embryo remained constant at 1.32 ± 0.22 μg. Therefore, the observed 50-fold increase in the percentage of tyrosinase RNA sequences reflects a real increase in the number of tyrosinase transcripts per embryo.

A comparison of the accumulation of tyrosinase RNA transcripts and the rate of tyrosinase protein synthesis throughout early embryogenesis in R. pipiens is presented in Fig. 5. Tyrosinase RNA values, expressed as the percentage of total embryonic RNA, were obtained from the hybridization data presented in Fig. 4. Values for the rate of tyrosinase protein synthesis were taken directly from the data of Benson and Triplett (7). The rate of tyrosinase synthesis was determined by comparing the amount of radiolabeled proteins present in anti-tyrosinase immunoprecipitates to that in trichloroacetic acid precipitates of embryos pulsed 6 h prior with a [3H]-amino-acid mixture. The coincidence of both the timing and amplitude of the tyrosinase RNA and tyrosinase protein inductive responses at the conclusion of gastrulation and through subsequent postinductive phases indicates that the translation of the tyrosinase zymogen is directly coupled to the accumulation of tyrosinase mRNA and that the accumulation of tyrosinase mRNA is dependent upon active transcription of the tyrosinase gene rather than the result of a processing of primary tyrosinase gene transcripts or an activation of masked tyrosinase mRNA.

**DISCUSSION**

The tyrosinase gene is a particularly interesting system for exploration of the molecular events underlying the control of gene expression during embryonic development. Our results...
Measurement of Tyrosinase mRNA Accumulation during Embryogenesis

TABLE I

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indicate that the percent of tyrosinase RNA transcripts in total embryonic RNA increased by a factor of at least 13.9 between the early gastrula (Stage 10–11) and late gastrula stages (Stage 12–13), coincident with the timing of neural induction (1–4) and the onset of tyrosinase zymogen synthesis (7). The accumulation of tyrosinase RNA closely paralleled the rate of tyrosinase zymogen synthesis throughout early embryogenesis; both underwent a near 50-fold amplification from the time of induction (Stage 12–13) to the heartbeat stage (Stage 19).

During the period of 50-fold accumulation in the percent of tyrosinase mRNA sequences, between Stages 11 and 19, the absolute number of dorsal ectoderm cells increased only by a factor of 7.3 and the percentage of dorsal ectoderm cells in the whole embryo remained relatively constant (30). Thus, the accumulation of tyrosinase mRNA sequences in postinductive embryos appears to reflect a corresponding increase in the number of tyrosinase mRNA sequences per neural crest cell rather than a proportionate increase in the number of neural crest cells per embryo. Accurate measurements of the number of neural crest cells in embryos has not as yet been accomplished; however, the use of [3H]tyrosinase cDNA to probe known quantities of purified neural ectoderm cells following treatment with soluble inducer molecules in vitro (31) should enable precise determination of the kinetics of tyrosinase mRNA induction on a per cell basis.

Based on the observations presented in this paper and in previous reports, we propose the following model, outlined in Table I, of the regulation of tyrosinase gene expression in *R. pipiens* embryogenesis. The phenomenon of gastrulation, Stage 10–12 (22–36 h), results in the morphological separation of the three primary germ layers. Determination of the neural crest in the dorsal ectoderm takes place at the conclusion of gastrulation, Stage 11–12 (29–36 h), in direct response to the inducing action of underlying chordamesoderm. Shortly thereafter, by Stage 12–13 (36–46 h), determination is manifest on a molecular level by the transcriptionally dependent accumulation of tyrosinase mRNA, nascent tyrosinase-bearing polysomes, and tyrosinase zymogen. Activation of the tyrosine oxidase moiety of the tyrosinase zymogen occurs 16 h later at Stage 15. This stage is also marked by the first appearance of active DOPA decarboxylase enzyme. Together, these events lead to the sequential synthesis of catecholamines in neural crest-derived chromaffin cells. Overt maturation of the melanophore component of the neural crest begins following the activation of the DOPA oxidase moiety of tyrosinase zymogen with the synthesis and deposition of melanin 80–100 h after determination, at Stage 20–21 (120–140 h).

The nature of the precise controls of tyrosinase mRNA accumulation, beyond its transcriptional dependence, remains open to a number of questions. For example, we cannot yet distinguish whether this accumulation is caused by an increased rate of tyrosinase mRNA synthesis or rather a stabilization of the mRNA against degradation. Purification of tyrosinase cDNA should now enable direct measurements of the rate of tyrosinase mRNA synthesis and of tyrosinase gene copy number and will permit a thorough exploitation of the
in vitro induction system which holds great promise for defining both the biochemistry and mechanism of action of soluble inducer molecules produced by chordamesoderm cells.

REFERENCES

Correcting for potential variations in technique, buffer and enzyme efficiency. The data overlaid with paraffin purified tyrosinase of 3H-tyrosinase CDNA to hybridization techniques. Reaction were incubated from 6-60 minutes to achieve the desired presence of 10-100ul reactions were conducted in precipitated with 2.5 ml 501 of reverse transcriptase and 2.5ug/ml of oligo dT 5'POlyA RNA or tyrosinase mRNA to yield a quick vortexed and incubated for five minutes at 4°C. The mixture was then adjusted to a final concentration of 60 nuclei/ml. The reaction was stopped by the addition of ten-volume of a 4% sodium-100 ml 10% trichloroacetic acid (TCA) and the mixture was centrifuged at 10,000 x g for 10 minutes. The precipitate was washed once with 95% ethanol and the RNA was extracted by heating to 60°C for five minutes and addition of 0.3 M NaCl, 0.3 M Na acetate and 1.20 Na borohydride. After 12 hours the precipitate was dissolved in 1 ml of 0.3 M Tris.HCl (pH 8.0) and the RNA was precipitated by the addition of 0.3 M NaCl, 0.3 M Na acetate and 2.5ug/ml of oligo dT. The RNA was collected by pipette, and rinsed thoroughly in distilled water. Tyrosinase CDNA was excised, packed into a 1.5 ml polypropylene microfuge tube and precipitated with 3.0 volumes of cold ethanol.

Hybridization Techniques

Hybridization of 3H-Tyrosinase CDNA to Tyrosinase mRNA. Hybridizations of 3H-Tyrosinase CDNA to Tyrosinase mRNA were performed in the presence of 100-1000 CPM of 3H-Tyrosinase CDNA in 26ul of reaction mix. Hybridizations were maintained at 60°C for 90 minutes. After hybridization, the RNA was washed in 2X SSC, 0.1% SDS at 60°C for 90 minutes. Hybridization washes were performed at 4°C for about 2 hours. That portion of the gel which corresponded to full-length 3H-Tyrosinase CDNA, as judged by parallel analysis on gel electrophoresis, was excised, packed into 1.5 ml polypropylene microfuge tubes and precipitated with 0.5 volumes of cold ethanol.

Measurement of Tyrosinase mRNA Accumulation during Embryogenesis

Tyrosinase mRNA Accumulation during Embryogenesis was evaluated by electrophoresis OD measurements. Developmental stages were dejellied with a 0.38 Na borohydride solution containing 3M NaCl and 0.01 M EDTA. The RNA was fully isolated from embryos. RNA concentrations were both synthesized. The former CDNA was complementary to total embryonic poly(A+ RNA and the latter CDNA was complementary to total embryonic poly(U RNA and to tyrosinase mRNA. The procedures for reverse transcription and hybridization with 3H-Tyrosinase CDNA are described in the preceding paper. Tyrosinase CDNA and 3H-Tyrosinase CDNA were reannealed at 60°C for 5 minutes. Reverse transcriptase was added to the ethanol precipitates of either RNA. RNAs were then adjusted to 35yM dATP, 80yM dCTP, 170yM dGTP and 200yM dTTP. The RNA was dissolved in 50 ul of stock solutions. The RNA was then adjusted to 0.3 M NaCl, 0.3 M Na acetate pH 5.51, mixed with a 0.3 M NaCl, 0.3 M Na acetate pH 5.51, and a 0.01 M EDTA, 10yM NaUH and the RNA was hybridized by heating to 60°C for 60 minutes.

Specific Activity

The specific activity of 1-2 ng/ml of RNA is expressed as percent of that isolated by the procedures of this report. Tyrosinase mRNA is expressed as percent of the full length of tyrosinase mRNA. The average size of length excised tyrosinase CDNA was 1200 nucleotides. Hybridization of 3H-Tyrosinase CDNA to Purified Tyrosinase mRNA was assessed from nuclei purified tyrosinase mRNA from early embryogenesis. Tyrosinase mRNA concentrations were obtained by hybridization of 3H-Tyrosinase CDNA with poly(U) RNA. Data containing 3H-Tyrosinase CDNA was used for the calibration of specific activity.

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