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

I. ISOLATION AND CHARACTERIZATION OF AMPHIBIAN TYROSINASE mRNA

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*Rana pippiens* tyrosinase mRNA was isolated from Stage 22 (tailfins circulating) embryos by indirect immunoprecipitation of embryonic polysomes using highly specific rabbit anti-tyrosinase and goat-anti-rabbit immunoglobulins. Analysis on sucrose gradients indicated that anti-tyrosinase bound specifically to embryonic polysomes of the 300–350 S class coincident with the location of nascent tyrosinase enzyme activity and tyrosinase mRNA. These same anti-tyrosinase-bound polysomes were fully immunoprecipitated by the addition of goat-anti-rabbit IgG.

Poly(A+) RNA was obtained from phenol-extracted antibody-polysome complexes by sequential passage over oligo(dT)-cellulose. The final purification of tyrosinase mRNA was achieved by preparative sucrose gradient fractionation.

Tyrosinase mRNA sedimented as a single 13 S peak in 5–30% sucrose gradients and tracked on sodium dodecyl sulfate-polyacrylamide gels as a single band of 4.5 x 10^6 Da (1275 nucleotides). When assayed in a cell-free translation system, this mRNA directed the synthesis of a single 35,000-Da protein which co-migrated with native tyrosinase on sodium dodecyl sulfate-polyacrylamide gels and which was >98% immunoprecipitable by anti-tyrosinase immunoglobulin. Final purification was 4103-fold over the starting polysomal RNA.

The induction and subsequent differentiation of neural crest cells in vertebrates provides an excellent model for observing the entire scope of events which transpire in the origin of a unique cell type. Studies on the amphibian neural crest have proven particularly fruitful. The embryology of neural crest induction in the frog, *Rana pippiens*, has been well defined; it is known that the juxtaposition of chordamesoderm and presumptive neural ectoderm during the midlate gastrula phase is crucial to neural crest induction and that chordamesoderm cells are capable of producing soluble inducer molecules when transplanted in vivo or cultured in vitro (1–7).

More recently, precise biochemical markers have been employed to define the developmental staging of neural crest derivatives. There is now good evidence that one of the early events of this staging is the appearance and activation of the bifunctional enzyme tyrosinase. It has been shown (8) that tyrosinase zymogen synthesis begins immediately following embryonic induction (at Stage 13) and that this is a transcriptionally dependent event. However, the nature of the molecular controls of tyrosinase gene expression at the time of embryonic induction remain to be determined and are the subject of this paper and its companion.

In this paper we first demonstrate that polysomes prepared from Stage 22 (tailfins circulating) embryos possess nascent tyrosinase enzyme activity and translatable tyrosinase mRNA in the 300–350 S region of sucrose gradients and that tyrosinase mRNA-bearing polysomes can be immunoprecipitated by anti-tyrosinase. We next report on the isolation of purified tyrosinase mRNA from immunoprecipitated polysomes and its characterization by both function and physical criteria.

**EXPERIMENTAL PROCEDURES AND RESULTS**

Purification and Characterization of Poly(A+) Tyrosinase mRNA from Stage 22 *R. pippiens* Embryos—Tyrosinase mRNA was isolated from Stage 22 *R. pippiens* embryos utilizing the reagents and procedures described previously in this paper. In summary: 1) tyrosinase mRNA-bearing polysomes were immunoprecipitated by anti-tyrosinase and goat-anti-rabbit immunoglobulin; 2) total RNA was isolated from antibody-polysome complexes by phenol/chloroform extraction; 3) poly(A+) RNA was isolated by chromatography on oligo(dT)-cellulose; and 4) poly(A+) RNA was enriched in tyrosinase mRNA by preparative sucrose gradient fractionation.

The extent of tyrosinase mRNA purification at each step of the isolation was assessed by monitoring RNA fractions for tyrosinase mRNA activity in a messenger-dependent, cell-free translation system. Tyrosinase mRNA activity was determined by measuring the quantity of [35S]methionine in the free translation system. Immunoprecipitates were also routinely examined on SDS-polyacrylamide gels to ensure the specificity of anti-tyrosinase precipitations. The pertinent quantitative comparisons of the purification scheme are shown in Table I and a summary of tyrosinase mRNA activity at each phase of the purification is presented in Fig. 5.

* Portions of this paper (including "Experimental Procedures," part of "Results," and Figs. 1–4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-285, cite the authors, and include a check or money order for $4.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

†The abbreviations used are: SDS, sodium dodecyl sulfate; DOPA, dihydroxyphenylalanine.

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TABLE I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>RNA (mg)</th>
<th>Total mRNA specific activity (cpm/µg)</th>
<th>Tyrosinase mRNA specific activity (cpm/µg)</th>
<th>Tyrosinase mRNA/Total mRNA</th>
<th>Purification Fold</th>
<th>Total tyrosinase mRNA activity (cpm x 10³)</th>
<th>Yield (%)</th>
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<tr>
<td>Total polysomal RNA</td>
<td>100</td>
<td>14,674</td>
<td>118</td>
<td>0.80</td>
<td>1</td>
<td>11.80</td>
<td>100</td>
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<td>Immunoprecipitate polyosomal RNA</td>
<td>0.536</td>
<td>14,777</td>
<td>9,664</td>
<td>65.49</td>
<td>1.9</td>
<td>5.18</td>
<td>44</td>
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<tr>
<td>Poly(A⁺) RNA</td>
<td>0.009</td>
<td>453,460</td>
<td>322,120</td>
<td>71.04</td>
<td>2731</td>
<td>3.19</td>
<td>27</td>
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<tr>
<td>Sucrose gradient peak</td>
<td>0.0036</td>
<td>490,821</td>
<td>484,130</td>
<td>98.64</td>
<td>4103</td>
<td>1.74</td>
<td>15</td>
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<td>Immunoprecipitate polysome supernatant</td>
<td>98.26</td>
<td>15,832</td>
<td>14</td>
<td>0.09</td>
<td>0.12</td>
<td>1.3</td>
<td>11</td>
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<tr>
<td>Poly(A⁻) RNA</td>
<td>0.485</td>
<td>7,621</td>
<td>2,445</td>
<td>32.08</td>
<td>20.72</td>
<td>1.19</td>
<td>10</td>
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</table>

The final purification of tyrosinase mRNA was then accomplished by selective isolation of unlabeled 13 S immunoprecipitated poly(A⁺) RNA from preparative sucrose gradients run in parallel. The success of this technique was first analyzed by electrophoresis of sucrose gradient-enriched [35S]poly(A⁺) RNA on a 4% polyacrylamide (98% formamide) gel (Fig. 6). The labeled RNA migrated as a single peak with a molecular weight of 4.5 x 10⁶, which corresponds to a nucleotide length of approximately 1275 bases. The unlabeled sucrose gradient peak RNA was analyzed in a cell-free wheat germ system for the ability to direct the de novo synthesis of tyrosinase. As shown in Table I, 98.64% of the total [35S]-labeled proteins synthesized were immunoprecipitable by anti-tyrosinase. This final purification step resulted in a 1.5-fold enrichment of tyrosinase mRNA activity in immunoprecipitated poly(A⁺) RNA and a 4103-fold purification from the total polysomal starting material.

Proof that purified tyrosinase mRNA was a faithful copy of the whole coding sequence was obtained by analysis of entire [35S]-labeled tyrosinase mRNA-primed wheat germ translation mixtures on polyacrylamide gels. As shown in Fig. 7, the endogenous incorporation of the wheat germ system was negligible (lane 1). Poly(A⁺) RNA³ prepared from total Stage 22 polysomes directed the synthesis of four prominent bands (M₉ > 150,000, 90,000, 70,000, and 20,000) and a scattering of less abundant species (lane 2). In agreement with previous results, 0.682% of labeled protein in this reaction was immunoprecipitated by anti-tyrosinase.

In reactions directed by anti-tyrosinase-immunoprecipitated poly(A⁺) RNA (lane 3), approximately 70% of the total [35S] incorporation was localized in a band which co-migrated with native tyrosinase monomer (M₉ = 35,000). This observation in conjunction with immunoprecipitation data and the

³ The use of Stage 22 poly(A⁺) RNA in these incubations as opposed to the true starting material for tyrosinase mRNA isolation, total Stage 22 polysomal RNA, was necessary to achieve levels of incorporation which could be resolved on polyacrylamide gels.
by parallel electrophoresis of radiolabeled reovirus on a 4% polyacrylamide-barbital (pH 9.0) tube gel containing 98% eukaryotic rRNA stained with ethidium bromide. Migration was from poly(A+) RNA digested, and then counted. The position of the molecular weight markers pictured in the upper portion of the figure were determined to the successful isolation of a full length tyrosinase mRNA. Sucrose gradient-purified digested, and then counted. The position of the molecular weight markers listed on the side of the figure are based on the published values for rabbit IgG light (23,900), heavy (50,000), and heavy-light chain dimer (68,000) and for R. pipiens epidermal tyrosinase (35,000). Electrophoresis was from top to bottom.

**FIG. 6.** Molecular weight determination of sucrose gradient-purified tyrosinase mRNA. Sucrose gradient-purified $^{125}$I-poly(A$^+$) RNA (10,000 cpm, approximately 6 ng) was electrophoresed on a 4% polyacrylamide-barbital (pH 9.0) tube gel containing 98% formamide. Following electrophoresis, the gel was frozen, sliced, digested, and then counted. The position of the molecular weight markers pictured in the upper portion of the figure were determined by parallel electrophoresis of radioactively labeled reovirus (REO) small, medium, and large RNAs and by UV-illumination of 28 S and 18 S eukaryotic rRNA stained with ethidium bromide. Migration was from left to right.

**Fig. 7.** SDS-polyacrylamide gel electrophoresis of products synthesized in the wheat germ system directed by different mRNA preparations. Translation reactions were carried out in 100-μl mixtures in the presence of 24 μCi of $[^{14}S]$methionine (600 Ci/mmole) for 90 min at 24°C. Reactions were directed by the addition of 1, no exogenous mRNA; 2, 0.5 μg of poly(A$^+$) RNA isolated from total Stage 22 R. pipiens embryos; 3, 0.5 μg of poly(A$^+$) RNA isolated from anti-tyrosinase-immunoprecipitated polysomes; and 4, 0.5 μg of sucrose gradient-enriched poly(A$^+$) RNA (tyrosinase mRNA). After incubation, 25-μl aliquots of the total reaction mixture were layered onto a 5%/10% discontinuous SDS-polyacrylamide slab gel and electrophoresed as described under "Experimental Procedures." Following electrophoresis, the gel was fixed, impregnated with an autoradiography enhancer, dried, and exposed to Kodak XR-1 film for 12 h at −70°C. The molecular weight markers listed on the side of the figure are based on the published values for rabbit IgG light (23,900), heavy (50,000), and heavy-light chain dimer (68,000) and for R. pipiens epidermal tyrosinase (35,000). Electrophoresis was from top to bottom. Vitro synthesis of native tyrosinase, which was unambiguously identified by electrophoretic mobility and immunoprecipitation analysis.

The final amount of tyrosinase mRNA obtained was 3.6 μg from 100 mg of Stage 22 polysomes starting material (45,000 tadpoles), with a yield of 15%. A significant percentage of the losses sustained during the purification was due to the sizable (23%) proportion of tyrosinase mRNA which failed to bind oligo(dT)-cellulose. A number of specific mRNAs have been detected in both the poly(A$^+$) and poly(A$^-$) populations (28, 29). Indeed, Kaufmann et al. (30) have observed that ribosome-bound poly(A$^+$) mRNAs represent a subset of sequences found in the poly(A$^+$) mRNA population. The significance of these observations and of poly(A$^-$) tyrosinase mRNA remains unknown. Other losses sustained during the purification are well within the standard ranges obtained by other workers using similar methodologies (28, 31–41). Thus, extensive losses due to ribonuclease degradation do not appear to have occurred.

The determination of the molecular weight of tyrosinase mRNA under denaturing conditions allows for calculation of the amount of nontranslated nucleotides. Tyrosinase mRNA has a molecular weight of $4.5 \times 10^6$ and therefore contains approximately 1275 bases. Tyrosinase has a molecular weight of 35,000 (10), corresponding to about 310 amino acids. Thus, approximately 930 nucleotides are required to code for tyrosinase leaving 345 nontranslated nucleotides. Nontranslated regions of this size are not uncommon in eukaryotic mRNAs. The size of nontranslated mRNA sequences reported in the literature range from a low of 90 bases to a high of nearly 1000, with the average being 200–500 in length (42).

**REFERENCES**

Isolation of Tyrosinase mRNA

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Isolation of Tyrosinase mRNA by Glen N. Gavlton and Edward L. Triplett

ExPERIMENTAL PROCEDURES

Isolation and characterization of embryonic tyrosinase RNA by Glen N. Gavlton and Edward L. Triplett

Experimental Procedures

Preparation of immunoglobulins. Immunocytological studies were directed to the purification of immunoglobulins from rabbit serum. The following procedure was used:

1. The serum was concentrated by diafiltration with an Amicon ultrafiltration system.

2. The concentrated serum was dialyzed against 0.01 M phosphate-buffered saline (pH 7.4) containing 0.15 M NaCl.

3. The dialyzed serum was applied to a column of Protein A-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with 0.01 M phosphate-buffered saline (pH 7.4) containing 0.15 M NaCl. The column was washed with 500 ml of the same buffer.

4. The immunoglobulins were eluted with 1 M acetic acid.

5. The eluate was dialyzed against 0.01 M phosphate-buffered saline (pH 7.4) containing 0.15 M NaCl.

6. The purified immunoglobulins were lyophilized and stored at -20°C.

Isolation of poliovirus RNA. Poliovirus RNA was isolated from poliovirus-infected HeLa cells as described by Griesenbach et al. (1974). The viral RNA was purified by the cesium chloride-polyethylene glycol density gradient centrifugation method.

Isolation of oligo(dT)-primed RNA. Oligo(dT)-primed RNA was isolated from HeLa cells by the method of Aviv and Hunkapiller (1975). The RNA was purified by the cesium chloride-polyethylene glycol density gradient centrifugation method.

Isolation of poly(A)-RNA. Poly(A)-RNA was isolated from poly(A)-rich RNA by the method of Aviv and Hunkapiller (1975). The RNA was purified by the cesium chloride-polyethylene glycol density gradient centrifugation method.

RESULTS

Embryonic Polysome Isolation

A procedure was utilized for the isolation of embryonic polysomes from 2500 Stage 22 Rana pipiens tadpoles. Eighteen such preparations were made, and the polysomes were then subjected to polyacrylamide gel electrophoresis. The polyacrylamide gel electrophoresis was performed by the method of Aviv and Hunkapiller (1975). The polysomes were then subjected to polyacrylamide gel electrophoresis.
Characterization of Nascent Tyrosinase Enzyme Activity and Tyrosinase Proteins on Electrophoretic Polyacrylamide

A demonstration of nascent tyrosinase activity associated with Stage 22 polysomes fractionated in 20-35% sucrose gradient is shown in Figure 2A. A peak of tyrosinase activity is clearly seen in the 25-35% sucrose region, coinciding with polysomes of the size which would be predicted to code for a polypeptide with Mr of 30,000-35,000 (25). The reported molecular weight of Rana pipiens epidermal tyrosinase is 35,000 daltons (10, 25), and the 2-5 fold increase over values reported previously (26) again emphasizes the benefits of the optimized polysome isolation procedure.

The specificity of anti-tyrosinase immunoprecipitation has been previously established (8,9). When incubated with homogenates of either adult or embryonic skin or whole embryos, precipitin curves were obtained using either purified adult tyrosinase (7). In addition, in immunoprecipitates of epidermal homogenates labeled in vivo with [35S]-methionine (90% of the total radioactivity in the 300-350s region) and 20% of the total tyrosinase activity located in the 300-350s region was precipitated in immunosolubination. Immunoprecipitation of nascent tyrosinase with purified anti-tyrosinase and protein A-Sepharose is presented in Figure 2B. The binding of [35S]-anti-tyrosinase to Stage 22 polysomes is presented in Figure 2A. The binding of [125I]-anti-tyrosinase to Stage 22 polysomes is presented in Figure 2A and B. The binding of [125I]-anti-tyrosinase to Stage 22 polysomes in the 300-350s region is indicated in Figure 2A and B. The binding of [125I]-anti-tyrosinase to Stage 22 polysomes in the 300-350s region is indicated in Figure 2A and B.

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