Development of the adrenergic phenotype: Increase in adrenal messenger RNA coding for phenylethanolamine-N-methyltransferase

(epinephrine/ontogeny/translation/gene expression/cell differentiation)

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ABSTRACT Mechanisms regulating the developmental increase in the activity of adrenal phenylethanolamine-N-methyltransferase (PNMTase), an index of the adrenergic phenotype, were examined. Immunoprecipitation indicated that the increase in catalytic activity in rat adrenal from birth to adulthood was attributable to increased numbers of PNMTase molecules, not enzyme activation. To determine whether the ontogenetic increase in PNMTase protein was associated with elevation of mRNA coding for PNMTase, cell-free translation was performed on total cellular mRNA obtained from adrenals at different ages. Translation in wheat-germ and reticulocyte lysate systems, followed by immunoprecipitation of the PNMTase product, NaDodSO₄ gel electrophoresis, and fluorography, showed an 8-fold increase in the proportion of specific PNMTase mRNA relative to total mRNA in rat adrenals from birth to adulthood. Moreover, bovine adrenal medullae exhibited a 100-fold increase in PNMTase mRNA levels between embryonic life and adulthood. Consequently, the ontogenetic increase in adrenal PNMTase appears to be due to a developmental rise in specific mRNA coding for the protein.

The adrenal medulla is a particularly useful model for defining the factors regulating expression and differentiation of the adrenergic phenotype because it is easily accessible, subject to hormonal influences, and expresses all the enzymes involved in epinephrine biosynthesis (1, 2). In the rat embryo at 13.5 days of gestation, medullary precursor cells, which have migrated from neural crest to adrenal primordium, contain the noradrenergic enzymes tyrosine hydroxylase (EC 1.14.16.20) and dopamine-β-hydroxylase (EC 1.14.17.1) (3–5). However, phenylethanolamine-N-methyltransferase (PNMTase; EC 2.1.1.28), the epinephrine-forming enzyme, is not detectable until 17.5 days of gestation, indicating that adrenergic differentiation occurs 4 days later than noradrenergic expression (4–6). Although adrenal glucocorticoid hormones are required for the developmental increase of PNMTase (6, 7), underlying molecular mechanisms are not known.

We find that the ontogenetic rise in PNMTase catalytic activity is accompanied by a parallel rise in enzyme protein. To determine whether the increase in numbers of PNMTase molecules is attributable to elevated levels of mRNA coding for the enzyme, we assayed rat and bovine adrenal mRNA for PNMTase in an in vitro translation system.

MATERIALS AND METHODS

Preparation of mRNA. Total cellular mRNA was prepared from the entire adrenal gland of Sprague–Dawley rats (Hilltop Animals, Scottsdale, PA) and from bovine (Holstein) adrenal medullae. The bovine adrenals were sent fresh on ice from Flow Laboratories (McLean, VA) and the medullae were dissected after arrival. For the isolation of mRNA, total cellular RNA was extracted with guanidine hydrochloride either by a modification of Cox's method (8, 9) or according to Liu et al. (10). The first method was used for initial experiments with bovine and rat adrenals; the second method was found to be more convenient, the mRNA was comparably active, and thus, it was used to compare PNMTase mRNA at various ages. The total RNA recovered per gram of tissue was similar at all ages examined. In all experiments, the mRNA was purified by oligo(dT)-cellulose chromatography (11) and the poly(A)-RNA was used for translation experiments. The A₂₆₀/A₂₈₀ ratio was at least 1.95.

Cell-Free Translations. The mRNA was translated with [³⁵S]methionine in a wheat-germ extract system at 25°C (12) or in a nuclease-treated rabbit reticulocyte lysate system at 37°C (13), as described (9). After incubation, an aliquot (5 µl) was taken to measure the formation of trichloroacetic acid-precipitable protein (14) and the remainder was used for immunoprecipitation.

Immunoprecipitation of PNMTase. The [³⁵S]methionine-labeled PNMTase was immunoprecipitated by a previously described (9) modification of Goldman and Blobel's method (15) and applied to a 6–12% gradient NaDodSO₄/polyacrylamide slab gel. Gel electrophoresis (16) was run according to a modification of the procedure of Maizel (17). The distribution of radioactive polypeptides and ¹⁴C molecular weight markers (Bethesda Research Laboratories) was determined by fluorography with sodium salicylate (18) on prefogged Kodak X-Omat AR5 film.

Radioactivity in the PNMTase immunoprecipitate was estimated by two methods. In the first, the x-ray pattern obtained by exposure of the gel to prefogged film was scanned on a Zeineh soft laser scanning densitometer (Biomed). The relative amounts of PNMTase synthesized in vitro were obtained by integration of areas under those peaks that corresponded to the molecular weight of PNMTase. In the second method, the radioactive bands were eluted according to Albanese and Goodman (19) and radioactivity was determined in 20 ml of Hydrofluor (National Diagnostics). A region of the gel without radioactive bands on the x-ray film was used as a blank.

Assay of PNMTase Activity. PNMTase catalytic activity was assayed by using minor modifications (20) of described methods (21). Protein was determined by the method of Lowry et al. (22).

Immunotitration. PNMTase immunotitration was performed by minor modifications of published methods (23). Adrenals were homogenized in 0.005 M Tris at pH 7.4, containing 1 mM dithiothreitol, trisylol at 100 units/ml, 0.2% bovine serum al-
bumin, and 0.2% Triton X-100. Homogenates were centrifuged at 6,000 \times g for 10 min, and 40 \mu l of the supernatant fraction was incubated with 20 \mu l of specific PNMTase antiserum, preimmune serum, or a combination thereof. After 1 hr of incubation at room temperature with 5 sec of vortexing every 15 min, the mixture was centrifuged at 48,000 \times g for 10 min. PNMTase catalytic activity was assayed in 20-\mu l aliquots of the supernatant fraction in duplicate.

**Histology.** To determine volume parameters, rat adrenal glands from rats at ages 1, 15, and 60 days (n = 4 at each age) were fixed by immersion in Bouin's fixative and embedded in paraffin. Serial 10-\mu m sections were stained with hematoxylin/eosin and projected with a Bausch and Lomb projector at \times 50 for day 1 and day 15 and at \times 20 for day 60; every fifth section was traced and the areas of cortex and medulla were measured with an electronic planimeter (Numonics). The total volumes of the adrenal cortex and medulla were calculated by integration of these areal measurements.

Immunofluorescence was performed on sections of bovine adrenal with Coons' method (24). Slices of bovine adrenal gland were fixed in 4% buffered paraformaldehyde and cryoprotected in 30% sucrose, and 10-\mu m sections were prepared for cryotomy. Preimmune serum was used as a control and in all cases gave negative staining.

**RESULTS**

**Ontogeny of PNMTase.** During development of the rat adrenal there is a dramatic increase in PNMTase catalytic activity (7, 20, 25). To assess whether this is a result of increased PNMTase molecule numbers or activation of preexistent enzyme, immunotitration of PNMTase from newborn and adult rats was performed (Fig. 1). Increasing amounts of antiserum were added to a fixed amount of enzyme antigen in solution. The adult preparation was diluted by a factor of 17 to approximate the concentration of PNMTase catalytic activity in the neonatal preparation. Immunotitration of neonatal and adult PNMTase yielded slopes that did not differ, suggesting that the postnatal increase in PNMTase activity is attributable to increased numbers of enzyme molecules. To determine, in turn,
FIG. 4. Effect of poly(A)-mRNA concentration on the cell-free synthesis of PNMTase. Rat adrenal poly(A)-mRNA was translated in the wheat-germ cell-free system. An aliquot (2 μl) was taken to determine the trichloroacetic acid-precipitable protein and the remainder (98 μl) was immunoprecipitated with antiserum against PNMTase and purified by NaDodSO4 gel electrophoresis. The 35S-labeled PNMTase was detected on x-ray film by fluorography. The film was aligned to the gel and the band corresponding to PNMTase was eluted and quantitated by scintillation spectrometry.

whether this increase in enzyme is accompanied by a corresponding increase in mRNA coding for PNMTase, we assessed the levels of PNMTase mRNA during development.

Translational Systems. Optimal conditions for translation of PNMTase mRNA and for immunoprecipitation were initially established with bovine adrenal medullary mRNA. Translation in a wheat-germ extract was linear for almost 60 min (Fig. 2A). The Mg2+ and K+ optima for translation were 1.2 mM Mg(OAc)2 and 112 mM KOAc, respectively (Fig. 2 B and C). However, 1.6 mM Mg(OAc)2 was preferable for translation of PNMTase mRNA, as shown in Fig. 2D. The newly synthesized polypeptide had an apparent Mr of 32,000.

These conditions were used for translation of rat adrenal mRNA. Immunoprecipitation with antisera to rat PNMTase yielded a [35S]methionine-labeled polypeptide with an apparent Mr of 35,000 (Fig. 3). Similar results were obtained with the rabbit reticulocyte lysate translation system.

To determine whether the immunoprecipitated PNMTase was, in fact, proportional to the amount of PNMTase mRNA present, varying concentrations of rat adrenal poly(A)-mRNA were used. Total translational activity and immunoprecipitable PNMTase were quantitated. Increased translation with increasing concentrations of adrenal poly(A)-mRNA was paralleled by increased immunoprecipitable PNMTase (Fig. 4). In each case, the PNMTase recovered was ~0.1% of the total translation product.

Ontogeny of Rat PNMTase mRNA. Developmental changes in PNMTase mRNA were investigated by isolating poly(A)-  

mRNA from rat adrenals on postnatal days 1 and 15 and from adults. The total translation products (Fig. 5) were strikingly similar, although not identical. (For example, a polypeptide of Mr 65,000 was greatly enriched in the translation of newborn mRNA.) To define developmental changes, an equal amount of total translation products at each age was immunoprecipitated with antisera to PNMTase. Whereas immunoprecipitates from adult mRNA exhibited a distinct PNMTase band, the comparable band was less intense at day 15 and barely detectable in the day 1 adrenals (Fig. 5). Quantitation of this change by elution of radioactive bands and scintillation spectroscopy indicated that PNMTase mRNA was enriched ~4-fold in the adult compared to the 15-day adrenal, whereas the newborn (day 1) was barely above background. Quantitation by densitometry gave comparable results for the increase in PNMTase mRNA from day 15 to adulthood and revealed an 8-fold increase from day 1 to adulthood (Table 1).

Because the entire rat adrenal gland was used in these studies, the total poly(A)-RNA fraction contained both cortical and medullary RNA. However, PNMTase is localized specifically to the medulla. To determine how the medulla changes in size relative to the cortex during development, we measured cortical and medullary volumes at days 1, 15, and 60. Although both divisions grew considerably, medullary volume, expressed as percentage of the total adrenal, remained relatively constant (Table 1).

Ontogeny of Bovine PNMTase mRNA. To eliminate the complicating factor of the cortex, we isolated bovine (Holstein) adrenal medullae from third-trimester embryonic, newborn, and adult animals. In embryos and neonates, there was a gra-

Table 1. Comparison of development of rat adrenal size, PNMTase activity, and PNMTase translation product

<table>
<thead>
<tr>
<th>Age</th>
<th>Cortical volume, mm³</th>
<th>Medullary volume, mm³</th>
<th>(Medullary volume/ adrenal volume)</th>
<th>PNMTase activity, nmol/pair/hr</th>
<th>Quantitation of PNMTase immunoprecipitate, % of adult</th>
<th>Densitometry</th>
<th>Elution and scintillation spectroscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>0.45 ± 0.02</td>
<td>0.029 ± 0.001</td>
<td>6</td>
<td>0.26 ± 0.01</td>
<td>12</td>
<td>0†</td>
<td>100</td>
</tr>
<tr>
<td>Day 15</td>
<td>1.95 ± 0.13</td>
<td>0.19 ± 0.02</td>
<td>9</td>
<td>4.3 ± 0.2</td>
<td>29</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Day 60 (Adult)</td>
<td>13.5 ± 0.5</td>
<td>0.8 ± 0.1</td>
<td>6</td>
<td>10.4 ± 0.3</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± SEM.
† Not significantly different from background.
dient of PNMTase immunoreactivity in the medulla with brightly stained cells located adjacent to the cortex and lightly stained cells located internally (Fig. 6). In the adult adrenal, most medullary cells were brightly stained for PNMTase.

PNMTase catalytic activity and translation of PNMTase mRNA, as a proportion of total translation products, increased markedly with age. Translation of PNMTase was extremely low in embryos and increased ~50-fold in the neonate and 100-fold in the adult (Fig. 7; Table 2).

**DISCUSSION**

Previous work has indicated that PNMTase, a specific index of the adrenergic phenotype, is initially expressed in the rat adrenal medulla late in fetal life (4–6). Although initial expression of PNMTase apparently occurs independently of high levels of glucocorticoids, the subsequent ontogenetic increase in catalytic activity and immunocytochemical reactivity is critically dependent on these steroid hormones (6, 7). The present studies demonstrate that the developmental rise in rat adrenal PNMTase activity is due to an increase in numbers of enzyme molecules and that development involves an increase in mRNA coding for PNMTase. Moreover, PNMTase mRNA increased during development of the bovine adrenal medulla, confirming results obtained in the rat whole adrenal.

Although the immunotitration studies demonstrated that the postnatal rise in rat adrenal PNMTase activity is attributable to an increase in specific enzyme protein and not to activation of preexistent enzyme molecules, it is not clear whether this increase reflects increased PNMTase per cell, increased cells, or both. It is likely that the observed increase between postnatal day 15 and adulthood is due to an increased amount of enzyme per cell because division of medullary cells has ceased completion by day 15 (26). In either case, the increased enzyme protein may be due to elevated rates of PNMTase synthesis or decreased degradation (or both).

One mechanism that might underlie increased PNMTase synthesis involves an ontogenetic increase in mRNA coding for PNMTase. To investigate this possibility, we used the method of cell-free translation to assess changes in PNMTase mRNA during development. Although such systems are sensitive to variations in salt concentrations and cannot be used to compare relative amounts of different mRNAs (27), they have been successful in evaluating varying amounts of the same mRNA during cell development (28). Direct quantitation of mRNA following in vitro translation has confirmed the reliability of this method (29).

A number of precautions were taken to ensure that developmental changes in mRNA detected did faithfully reflect the amounts of PNMTase mRNA present. First, optimal conditions for translation of PNMTase mRNA were established (Figs. 2 and 3). Second, control experiments indicated that increasing amounts of poly(A)-RNA in the translation system yielded a nearly linear increase in the PNMTase precipitated (Fig. 4). Third, total cellular mRNA was employed to ensure that we assessed the total mRNA and not merely a potential subpopu-

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**Table 2. PNMTase development in bovine adrenal medulla**

<table>
<thead>
<tr>
<th>Age</th>
<th>PNMTase activity, pmol/μg of protein/hr</th>
<th>Quantitation of PNMTase translation product, % of adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>Third-trimester embryos*</td>
<td>0.90 ± 0.14</td>
<td>1–3</td>
</tr>
<tr>
<td>Newborn</td>
<td>1.8 ± 0.4</td>
<td>55</td>
</tr>
<tr>
<td>Adult</td>
<td>11.7 ± 1.2</td>
<td>100</td>
</tr>
</tbody>
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* Mean ± SEM.
† Denasitometric method.
‡ Slaughterhouse specimens for which precise ages were not available.
lation functionally active on polysomes. Fourth, the apparent molecular weight of the synthesized, precipitated PNMTase of 35,000 in the rat and 32,000 in the cow are in agreement with others (30–32). [We have not yet identified an additional polypeptide, with apparent Mr of 48,000 (Fig. 5), which inconsistently appeared in immunoprecipitates of rat adrenal preparations.] Fifth, two independent methods were used to quantitate the PNMTase translation product, laser densitometry and scintillation spectroscopy, yielding comparable results. Finally, developmental comparisons were made only within single experiments to ensure comparable efficacy of immunoprecipitation and translational efficiency.

Our results indicate that rat adrenal poly(A)-mRNA becomes enriched for PNMTase message during development. Consequently, the ontogenetic increase of PNMTase in vivo appears to be due to increased specific mRNA leading to enhanced PNMTase synthesis. This increase may be due to increased specific mRNA per cell or an increased number of cells producing message (or both). However, interpretation is complicated by the fact that assays in the rat contained both cortex and medulla. Nevertheless, because the ratio of medullary and cortical volumes was unchanged during development (Table 1), it is unlikely that the observed increases in PNMTase mRNA were due to decreases in cortical total poly(A)-mRNA. To investigate this point, we assayed PNMTase mRNA from pure medullary preparations of bovine adrenals at different ages. Bovine medullary PNMTase mRNA increased during development as in the rat, suggesting that this is a generalized phenomenon. However, in the bovine system, the increases in PNMTase activity and in mRNA were not completely parallel: message increases prior to enzyme activity.

The developmental increase of PNMTase mRNA could result from a decreased rate of degradation (8, 33–35). Alternatively, the biosynthesis of PNMTase mRNA may be increased through several different mechanisms, including (i) stimulation of gene expression (36), (ii) enhanced processing of heterogeneous nuclear RNA, increasing the poly(A)-mRNA coding for PNMTase, or (iii) increased export of the mRNA into the cytoplasm (37).

Regardless of underlying mechanisms, our observations demonstrate that the developmental increase in adrenomedullary PNMTase is accompanied by increased PNMTase mRNA. Since the development of PNMTase is glucocorticoid-dependent, it is possible that glucocorticoids regulate the developmental increase in PNMTase mRNA. This suggestion is consistent with the observation that glucocorticoids increase specific mRNA for tyrosine hydroxylase in pheochromocytoma (38), for tyrosine aminotransferase and tryptophan oxygenase in rat liver (39, 40), for phosphoenolpyruvate carboxykinase in rat kidney (41), and for growth hormone in cultured pituitary cells (42). Moreover, since glucocorticoids affect the development of PNMTase in extraadrenal loci, such as sympathetic ganglia (20, 21, 43), a generalized influence of these hormones on PNMTase mRNA in various locations cannot be excluded.

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