Hormonal Control of Rat Adrenal Phenylethanolamine N-Methyltransferase

Enzyme Activity, the Final Critical Pathway

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To examine whether glucocorticoids control rat adrenal phenylethanolamine N-methyltransferase (PNMT) through gene transcription, the effects of hypophysectomy and acute and chronic glucocorticoid replacement on PNMT mRNA and enzymatic activity were determined. Glucocorticoid depletion through hypophysectomy did not alter PNMT mRNA, whereas PNMT activity declined to ~25% of normal. A single dose of ACTH (4 IU SC) rapidly induced PNMT mRNA, with a six-fold peak at 6 hours postinjection. The short-term rise in PNMT mRNA was accompanied by an increase in corticosterone and elevated levels of glucocorticoid receptor mRNA. Ribosomal loading experiments suggested that available PNMT mRNA was fully utilized for protein synthesis. However, PNMT activity did not increase commensurately. Chronic ACTH treatment (4 IU SC daily for 7 days) sustained elevated levels of glucocorticoid receptor mRNA

but returned corticosterone to hypophysectomized levels and decreased PNMT mRNA to 50% of normal. Despite the decline in PNMT mRNA and its partial utilization for protein synthesis, PNMT enzymatic activity was fully restored. These findings indicate that glucocorticoids exert marked but complex influences on PNMT gene transcription. In addition, corticosteroids appear to posttranscriptionally regulate PNMT protein expression, underscoring the uncoupling between the expression of PNMT mRNA and active enzyme. Thus, glucocorticoid control of gene transcription and protein synthesis do not fully account for changes in PNMT expression, consistent with the previous observation that glucocorticoid control of PNMT proteolysis is also important in PNMT regulation and the potential for epinephrine biosynthesis. [Neuropsychopharmacology 13:223–234, 1995]

The catecholamine biosynthetic enzyme phenylethanolamine N-methyltransferase (PNMT, E.C. 2.1.1.28), the final enzyme in the pathway converting tyrosine to epi-nephrine, is a critical determinant of epinephrine expression during acute and chronic stress. In response to stress, large quantities of glucocorticoids and epinephrine are released from the adrenal gland. Further, glucocorticoids have been demonstrated to affect the expression of PNMT, and it has been suggested that the primary source of this control occurs through PNMT gene expression (Evinger et al. 1992). However, the mechanism(s) by which hormones regulate PNMT still remains a subject of much controversy.

Early studies in the adult rat showed that depletion of glucocorticoids through hypophysectomy markedly reduced PNMT enzymatic activity to ≤20% of normal values (Ciaranello et al. 1975; Wong et al. 1985; Evinger et al. 1992). Basal levels of the enzyme could be restored by adrenocorticotropin (ACTH) or glucocorticoid ad-

ministration. However, PNMT enzymatic activity could never be elevated beyond normal through corticosteroid manipulation (Ciaranello et al. 1975). Protein turnover studies indicated that hormones exerted their effects by controlling the rate of protein degradation without affecting protein synthesis (Ciaranello et al. 1975). In the presence of corticosteroids, levels of the cosubstrate S-adenosylmethionine (SAM), the methyl donor for the enzymatic reaction catalyzed by PNMT, were sustained, and the binding of SAM to PNMT protected the enzyme against proteolytic degradation (Ciaranello 1978; Berenbeim et al. 1979; Wong et al. 1982; Wong et al. 1985).

More recently it has been demonstrated that PNMT gene expression is also hormonally regulated. Whereas the effects of hypophysectomy on PNMT mRNA have been variable, no change (Wong et al. unpublished; Evinger et al. 1992) or marked depletion (Jiang et al. 1989), corticosteroids have consistently been shown to restore (Jiang et al. 1989) and/or elevate PNMT mRNA beyond normal levels (Wong et al. 1992; Evinger et al. 1992) in the adult rat.

Glucocorticoid control of PNMT transcription is consistent with the presence of a glucocorticoid response element (GRE) in the 5' upstream promoter/regulatory sequences of the PNMT gene (Baetge et al. 1988; Kaneda et al. 1988; Batter et al. 1988; Ross et al. 1990; Morita et al. 1992). Furthermore, the rat GRE appears functional based on the induction of chloramphenicol acetyltransferase (CAT) reporter gene activity from a PNMT-CAT reporter gene construct transiently expressed in bovine chromaffin cells (Ross et al. 1990). However, the induction of reporter gene expression by dexamethasone occurred only in cells expressing the endogenous PNMT gene and required very high corticosteroid concentrations (20 μM).

This observation, coupled with evidence demonstrating that corticosteroids are unable to initiate PNMT expression prematurely in the developing rat adrenal gland or cultured pheochromoblasts (Bohn et al. 1981; Michelson and Anderson 1992), suggested that glucocorticoids might be incapable of independently activating the PNMT gene. Rather, a transcriptional factor, other than the glucocorticoid receptor (GR), might be necessary to initiate PNMT gene expression, a factor with which the activated GR could then interact to facilitate PNMT gene transcription.

Consistent with this hypothesis, we have shown recently using transient cotransfection assays that the immediate early gene transcriptional protein, Egr-1 (Sukhatme et al. 1988), activates both a PNMT promoter-luciferase reporter gene construct and the endogenous PNMT gene in a PC12 derivative cell line, RS1 (Ebert et al. 1994). Further, dexamethasone (1 μM) induces an additional two-fold increment in both reporter

gene and endogenous PNMT gene expression, although dexamethasone, by itself, does not appear to activate either the PNMT-reporter gene construct or the endogenous PNMT gene. Deletion mutation and site directed mutagenesis studies demonstrated that Egr-1 activation is mediated through two Egr-1 consensus sequences, a perfect match sequence and an 8/9 match sequence (-165 bp and -45 bp upstream of the site of transcription initiation), whereas the glucocorticoid response is mediated through the GRE (-513 bp) in the 5' upstream PNMT promoter/regulatory region.

It is also evident from in vivo studies that PNMT transcriptional activity is likely uncoupled from PNMT translational activity. For example, when intact rats were administered the type II specific glucocorticoid agonist, RU28362, or the mixed type I and type II glucocorticoid agonist, dexamethasone, PNMT enzymatic activity declined, falling to 70% of normal levels at a dose of 30 μg daily of dexamethasone or 100 μg daily of RU28362 (Wong et al. 1992). Whereas higher doses of RU28362 sustained the attenuation of PNMT, higher doses of dexamethasone restored PNMT activity to normal. PNMT mRNA showed a markedly different pattern. Whereas low doses of RU28362 (100 μg daily) did not change PNMT mRNA levels, high doses ($\geqslant$100 μg daily) elevated mRNA levels as much as eleven-fold (1,000 μg daily). A similar pattern was observed with dexamethasone except that the rise in PNMT mRNA occurred at a lower dose (30 μg daily) and the increment in mRNA expression was much greater ($\sim$20 fold at 1,000 μg daily).

The present studies were undertaken to further examine the apparent independent effects of glucocorticoids on PNMT gene and protein expression using a paradigm of glucocorticoid depletion and restoration and to determine the relative importance of these mechanisms in controlling the critical biologic endpoint, PNMT enzymatic activity. Our findings confirm the uncoupling of glucocorticoid control of PNMT mRNA and protein expression. In response to glucocorticoid depletion through hypophysectomy, PNMT mRNA expression was not significantly reduced, whereas PNMT enzymatic activity markedly declined. Acute corticosteroid replacement rapidly elevated PNMT mRNA well beyond normal levels without a concommitant elevation in PNMT enzymatic activity. In contrast, chronic glucocorticoid replacement therapy restored PNMT enzymatic activity to normal levels although PNMT mRNA was reduced to half of normal values. Although PNMT mRNA appeared fully and comparably utilized for protein translation in the case of acute corticosteroid replacement, it did not appear to be fully utilized in the case of chronic replacement therapy. Clearly, PNMT gene transcription alone is not the limiting event in PNMT enzymatic expression.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (175 to 200 g), obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN), were acclimated to a 12:12 dark/light cycle (lights on at 7 A.M.) and provided with food and water or 1/4 normal plasma salts (hypophysectomized rats) ad libitum. Hypophysectomy was performed by the vendor. Each experiment consisted of intact, ACTH-treated hypophysectomized rats as indicated with at least six animals per treatment group. ACTH (H.P. ACTHAR GEL, 40 IU/ ml, diluted to 16 IU/ml with 16% gelatin, Rorer Pharmaceutical Corp, Fort Washington, VA) was administered subcutaneously at a dose of 4 IU (0.25 ml) per injection. For acute treatment, a single dose was administered, and animals were killed at 0, 3, 6, 12, and 24 hours posttreatment. For chronic studies, animals received 4 IU of ACTH SC daily for 7 days and were sacrificed at 6 or 12 hours after the final injection. Untreated intact and hypophysectomized rats were used as controls as we have found no significant differences between untreated and vehicle treated (0.25 ml of 16% gelatin) animals. After decapitation, adrenal glands were removed, quick frozen on dry ice, and stored at −80°C. The left adrenal gland was used for enzyme activity measurements and the right adrenal gland for mRNA quantitation.

Corticosterone

As previously reported (Wong et al. 1992), adrenal corticosterone was determined by radioimmunoassay (ICN Biomedicals, Inc., Costa Mesa, CA). Two dilutions of every sample were measured to verify the corticosterone content.

RNase Protection Assay

Total RNA was isolated from the right adrenal gland as previously described (Wong et al. 1992). For each PNMT mRNA protection assay, 3 µg of total RNA was hybridized simultaneously with a [^{32}P]PNMT cRNA probe (2 × 10^5 dpm, specific activity 1 × 10^8 dpm/µg) and a β-actin cRNA probe (1 × 10^4 dpm, specificity 1 × 10^8 dpm/µg) (Wong et al. 1993). For GR mRNA quantitation, 5 µg of total RNA was simultaneously hybridized with a [^{32}P]GR cRNA probe (1 × 10^5 dpm, specific activity 1 × 10^8 dpm/µg) and a [^{32}P]glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cRNA probe (5 × 10^3 dpm, specific activity 1 × 10^6 dpm/µg, Ambion, Austin, TX) (Wong and Wang 1994). Protected PNMT, β-actin, GR, and GAPDH mRNA bands were quantitated from the gel autoradiograms by computerized densitometry (Wong and Wang 1994), using

[^{35}S]PNMT and [^{35}S]GR sense mRNA as calibration standards (1–60 pg). PNMT and GR mRNA were then normalized against β-actin and GAPDH mRNA respectively and expressed as relative OD units.

PNMT Assay

PNMT enzymatic activity was measured in duplicate by quantitating the amount of radiolabeled, methylated product formed from phenylethanolamine and [^{3}H]S-adenosylmethionine (Wong et al. 1992).

Protein

Adrenal protein was quantitated using the Biorad protein assay (Biorad, Richmond, CA) and bovine immunoglobulin G (Sigma, St. Louis, MO) as the calibration standard.

Ribosomal Loading

Ribosomal loading experiments were performed on pooled adrenal pairs from intact (six pairs), ACTH-treated intact (six pairs), hypophysectomized (12 pairs), and ACTH-treated hypophysectomized (12 pairs) rats as indicated (Wong and Wang 1994). Polysomes were isolated and then fractionated on a linear sucrose gradient (0.5 to 1.5 M sucrose) by centrifugation at 270,000 × g for 110 min at 4°C. Fractions of 0.6 ml were collected and the A$_{260}$ determined for each. Following phenol:chloroform extraction and ethanol precipitation, the total RNA from each fraction was resuspended in 30 µl (intact and ACTH-treated intact rats) or 17 µl (hypophysectomized and ACTH-treated hypophysectomized rats) of 10 mM Tris-HCl, pH 7.5/0.1 mM EDTA, pH 8.0. The A$_{260}$ was then redetermined for each fraction and a 10 µl aliquot analyzed for PNMT mRNA by RNase protection assay as described earlier.

Statistical Methods

CORT, mRNA, and PNMT activity are expressed as the mean for at least five to six animals ± SEM. Statistical significance was determined using the two-tailed Student's t-test. As described previously, relative PNMT and GR mRNA values were calculated by normalization to β-actin and GAPDH mRNA respectively. All experiments were validated at least two to three times.

RESULTS

Corticosterone

ACTH stimulates the synthesis of the endogenous glucocorticoid, corticosterone, in the adrenal cortex. To examine the effects of ACTH treatment on glucocorticoid
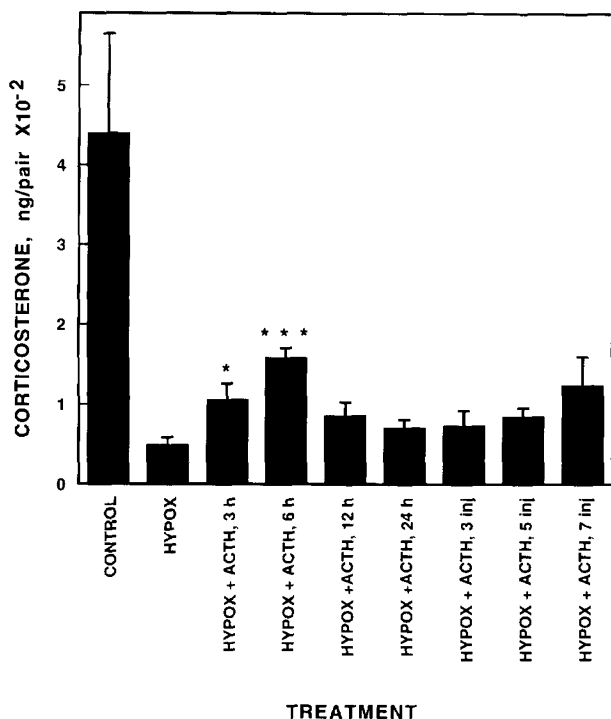
Figure 1. Adrenal corticosterone levels in intact, hypophysectomized, and ACTH-treated hypophysectomized rats. Hypophysectomized male Sprague Dawley rats (175 to 200 g) were administered 4 IU of ACTH SC. For acute studies, animals were administered one dose of the drug and killed at 3, 6, 12, or 24 hours after injection. For chronic studies, ACTH administration was continued on a daily basis and animals killed 24 hours after the third, fifth, or seventh injections. Adrenal glands were removed and corticosterone concentrations determined by RIA (ICN Biomedicals, Inc., Costa Mesa, CA) using the left adrenal gland as described in the Materials and Methods section. Adrenal glands from vehicle-injected intact and hypophysectomized animals provided basal and depleted corticosterone levels. Corticosterone is expressed as the mean ± SEM, with six animals per group and the corticosterone level for each animal representing the average determined from two tissue dilutions lying within the linearity range of the RIA. Significantly different from hypophysectomized control; * $p \leq .05$; *** $p \leq 10^{-4}$.

replacement in hypophysectomized animals, hypophysectomized rats were administered an acute dose of ACTH (4 IU SC) and killed at 0, 3, 6, 12, or 24 hours or were chronically treated (4 IU SC daily) and killed 24 hours after three, five, or seven injections of ACTH, and adrenal corticosterone was quantitated by radioimmunoassay.

After hypophysectomy, adrenal corticosterone (CORT) concentrations markedly declined to 10% of control values (Figure 1). A single dose of ACTH restored CORT but only partially. The response was rapid so that by 3 hours, adrenal CORT levels were two-fold

($p = .02$) that observed in the untreated hypophysectomized animal. The maximum rise in CORT occurred at 6 hours (3.6-fold hypophysectomized values, $p < 10^{-4}$), but peak concentrations represented only 40% of normal levels. After 6 hours, CORT declined, reaching the levels observed in hypophysectomized animals by 24 hours postinjection. In addition, chronic ACTH treatment (4 IU SC daily for up to 7 days) did not significantly elevate steady-state levels of adrenal CORT in the hypophysectomized animals even after 7 days of treatment.

These results suggest that the response of CORT to ACTH priming may be biphasic. An acute dose of ACTH appeared to initiate a rapid restoration of endogenous CORT but did not completely restore CORT to normal levels nor sustain its elevation. Moreover, chronic ACTH treatment appeared both incapable of elevating or restoring steady-state levels of CORT to normal values, although we cannot exclude the possibility that ACTH may initiate a transient rise in CORT.

PNMT and Glucocorticoid Receptor mRNA

To examine whether PNMT mRNA expression exhibited a pattern consistent with a glucocorticoid inducible gene, PNMT and GR mRNA were quantitated for each of the treatment groups in the acute and chronic ACTH-hypophysectomy paradigm. As shown in Figure 2, adrenal PNMT mRNA was not significantly different in the untreated hypophysectomized and intact rats. Similar to CORT, PNMT mRNA rapidly rose (∼six-fold control, $p \leq 10^{-4}$) after the initiation of ACTH treatment, and also peaked at 6 hours. After 12 hours, PNMT mRNA declined, approaching near normal levels by 24 hours (1.5-fold control, $p = .05$), but then decreased further to 50% of normal levels with chronic ACTH treatment ($p = .05$).

Figure 2 also depicts concurrent changes in adrenal GR mRNA expression. Hypophysectomy caused an ∼three-fold increment in GR mRNA ($p \leq 10^{-3}$). In response to ACTH treatment, GR mRNA began to decrease, reaching normal values at 12 hours after an acute dose of ACTH ($p = .92$). Thereafter, GR mRNA rose again, but never beyond 1.6-fold control levels ($p \leq .04$), even with chronic treatment.

The coincident peak in CORT and PNMT mRNA is consistent with the PNMT gene being glucocorticoid responsive. Although the rise in GR mRNA suggests a rapid compensatory decline in response to glucocorticoid replacement, GR mRNA was still 60% above control levels at the time when both CORT and PNMT mRNA peaked, again consistent with acute corticosteroid treatment being mediated through PNMT gene induction. In contrast, with chronic steroid replacement therapy, GR mRNA was elevated whereas CORT and PNMT mRNA were reduced, suggesting that in the lat-
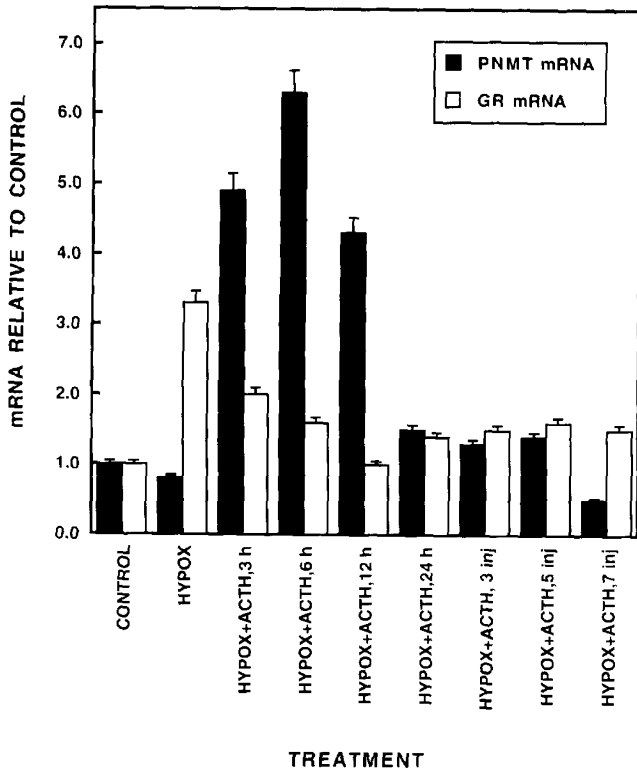
Figure 2. Adrenal PNMT and GR mRNA levels in intact, hypophysectomized, and ACTH-treated hypophysectomized rats. Rats were treated as described in Figure 1 and total RNA prepared from the right adrenal gland as described in the Materials and Methods section. RNase protection assays were performed on each sample as previously described (Wong et al. 1993; Wong and Wang 1994).To measure PNMT mRNA, 3 µg of total RNA was simultaneously hybridized with a [32P]PNMT cRNA probe (2 × 10^5 dpm, specific activity 1 × 10^8 dpm/µg) and a [32P]β-actin cRNA probe (1 × 10^{-4} dpm, specific activity 1 × 10^5 dpm/µg). To measure GR mRNA, 5 µg of total RNA was simultaneously hybridized with a [32P]GR cRNA probe (1 × 10^5 dpm, specific activity 1 × 10^8 dpm/µg) and a [32P]GAPDH cRNA probe (5 × 10^3 dpm, specific activity 1 × 10^6 dpm/µg). For each sample, PNMT and GR mRNA were normalized respectively to their β-actin and GAPDH mRNA controls. mRMA concentrations are expressed relative to levels in adrenal glands from intact animals. Values represent the mean ± SEM of six animals.

ter case, glucocorticoid control may not be mediated through control of PNMT gene expression.

PNMT Activity

Although it has always been assumed that transcriptional changes specify translational changes, accumulating evidence suggests that changes in transcription and translation may not always be concordant. To determine whether glucocorticoids evoke corresponding

changes in PNMT enzymatic activity and PNMT mRNA, PNMT activity was measured in the adrenal gland of intact, hypophysectomized, and ACTH-treated hypophysectomized rats. As shown in Figure 3, after hypophysectomy, enzyme activity declined to ~26% of that observed for intact animals. ACTH treatment of hypophysectomized rats initiated a slow restoration in PNMT activity so that with an acute dose of the drug, activity was restored to 44% of intact levels (170% of hypophysectomized levels) by 24 hours. However, basal (intact) levels of the enzyme were not restored until the animals had received the full chronic regimen of ACTH (4 IU SC daily for 7 days).

These results indicate that PNMT transcription and translation are likely uncoupled. Whereas PNMT mRNA was maximally induced at 6 hours after an acute dose of ACTH, normal levels of PNMT enzymatic activity could only be restored by long-term ACTH exposure, when PNMT mRNA was, in fact, ~50% of its levels in the intact rat.

Ribosomal Loading

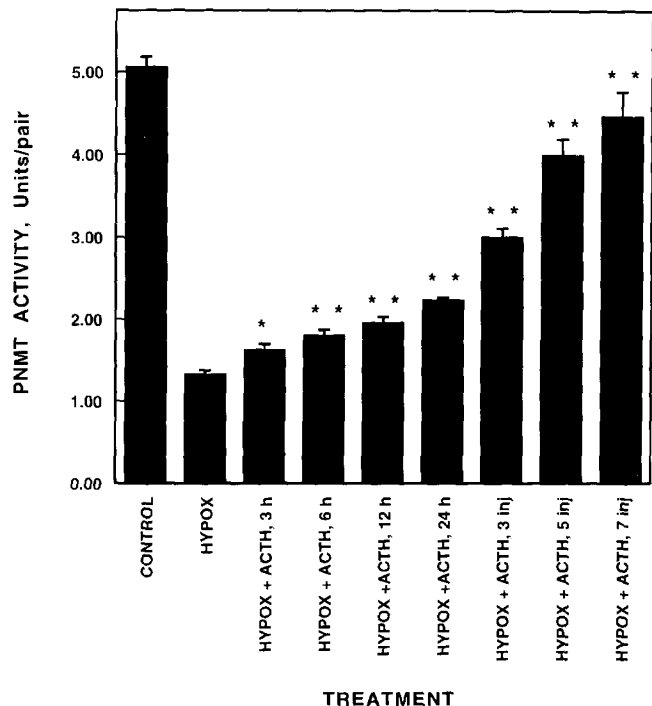Ribosomal loading experiments were used to examine whether the discordant changes in PNMT mRNA and

Figure 3. Adrenal PNMT activity in intact, hypophysectomized, and ACTH-treated hypophysectomized rats. Rats were treated as described in Figure 1 and the left adrenal gland from each animal assayed for PNMT activity as previously described (Wong et al. 1992). Activity is represented as the mean ± SEM with six animals per group. Significantly different from hypophysectomized control; * $p \le .05$, ** $p \le 10^{-3}$.
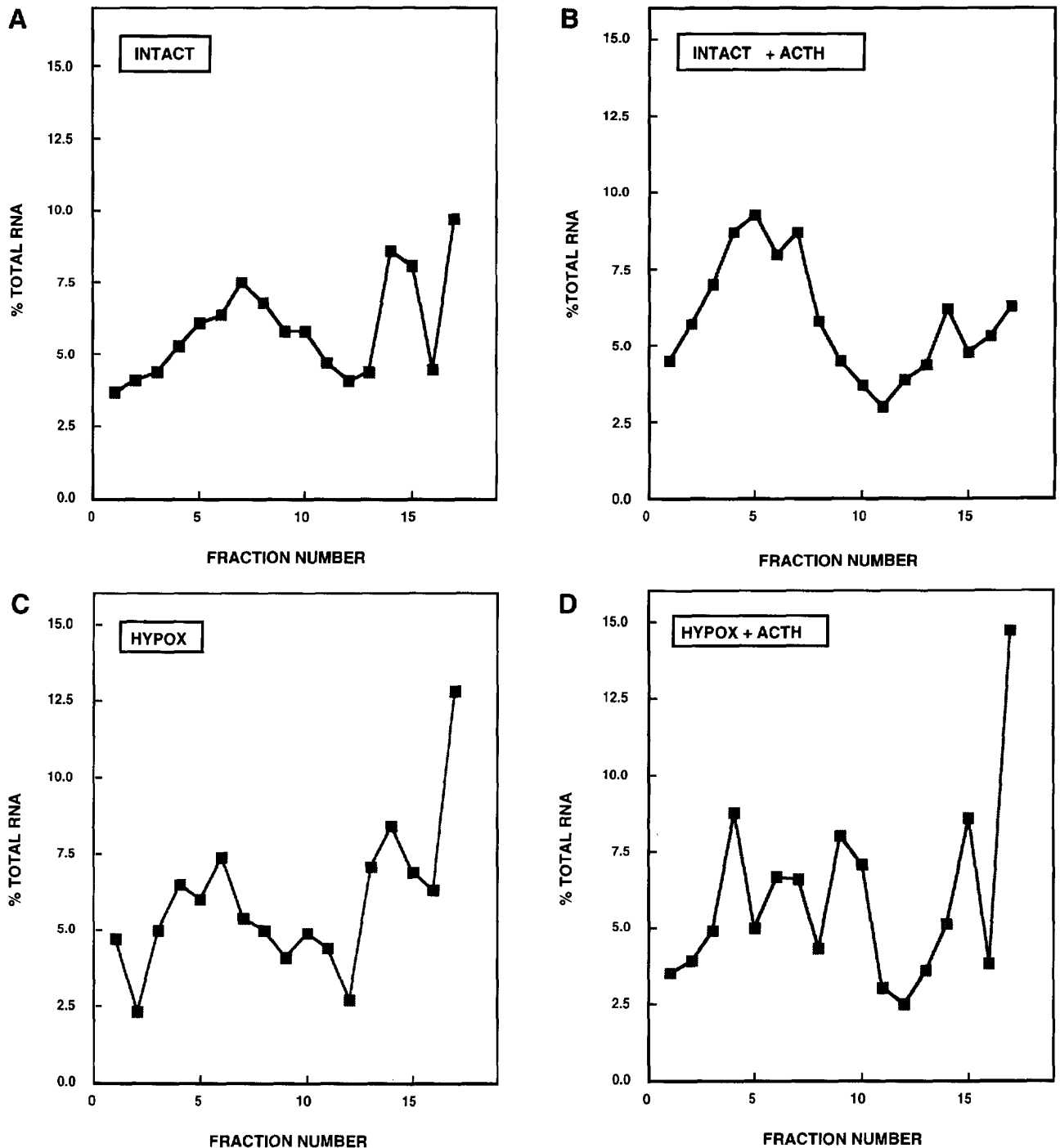
Figure 4. Polysome distribution, acute ACTH treatment. Intact and hypophysectomized rats were administered a single dose of ACTH (4 IU SC) and killed at 3, 6, 12, and 24 hours postinjection. Controls include vehicle-injected intact and hypophysectomized animals. Polysomes, prepared from the adrenal glands, were separated on a linear sucrose gradient (0.5 to 1.5 M) as described in the Materials and Methods section. Fractions of 0.6 ml were collected and the A_{260} determined for each to generate the polysome distribution profiles shown. (A), intact; (B), ACTH-treated intact; (C), hypophysectomized; and (D), ACTH-treated hypophysectomized.

enzyme activity could be attributed to differences in the utilization of PNMT mRNA for protein synthesis. Polysomes isolated from the adrenal glands of intact, hypophysectomized, and ACTH-treated intact or hy-pophysectomized animals receiving a single dose of ACTH were separated on a linear sucrose gradient (0.5 to 1.5 M sucrose) and the gradient fractions analyzed for RNA content based on their A_{260} values (Figure 4).
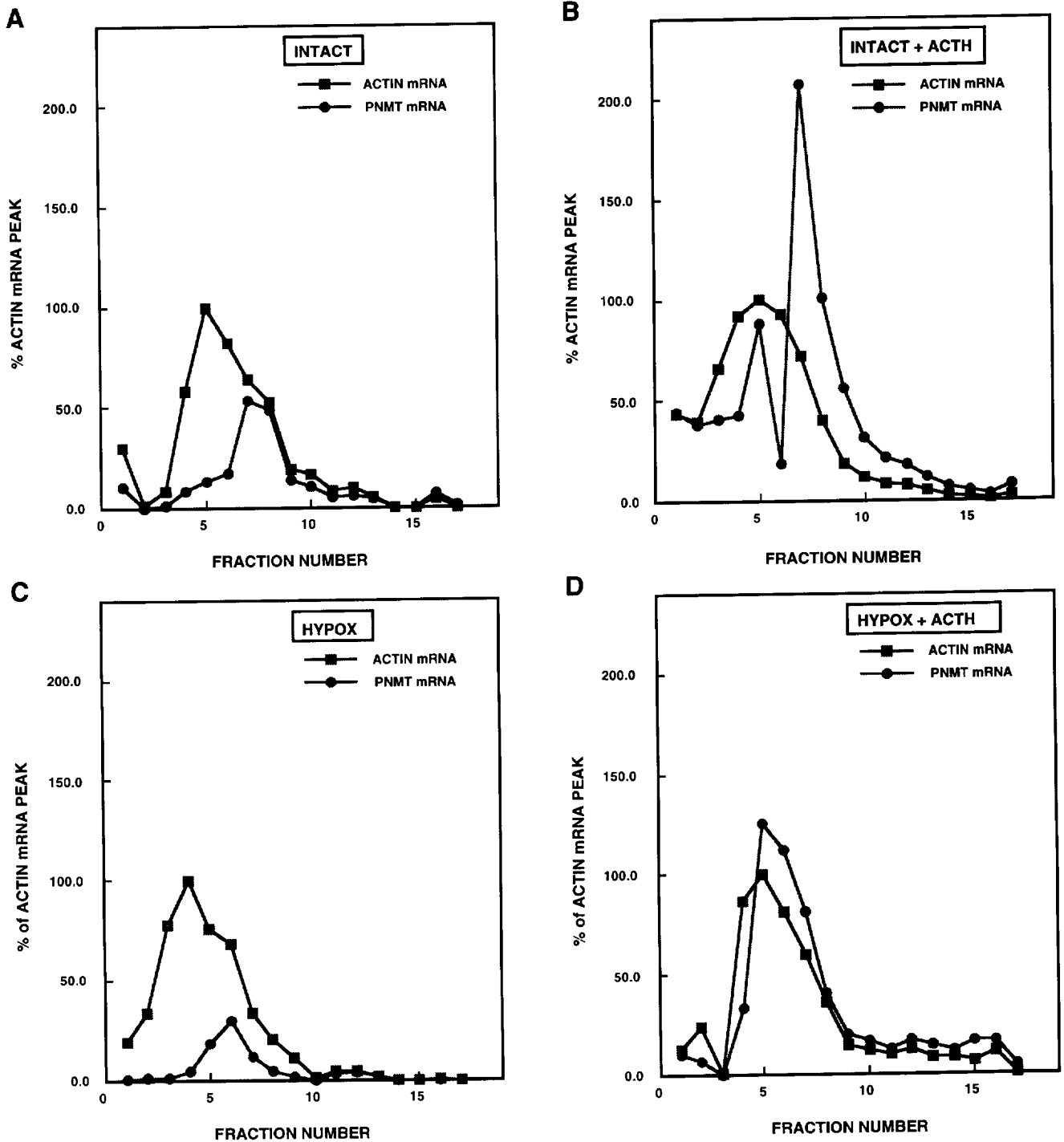
Figure 5. Utilization of PNMT mRNA pool, acute ACTH treatment. Each polysome fraction shown in Figure 4 was analyzed for PNMT mRNA by RNase protection assay as described in the Materials and Methods section. mRNA levels in each fraction are expressed relative to the peak β-actin fraction (100%) for the treatment group. (A), intact; (B), ACTH-treated intact; (C), hypophysectomized; and (D), ACTH-treated hypophysectomized.

As gradient densities decrease from left to right, the early emerging fractions (≤10) likely represent ≥trisomes and the late emerging fractions (≥12) likely represent monosomes or disomes. Although the polysome patterns were complex, there appeared to be an incre-

ment in the ≥trisome and the monosome/disome pools in both the intact and hypophysectomized animals administered a single dose of ACTH. When each fraction was subsequently analyzed for PNMT and β-actin mRNA content by RNase protection assay (Figure 5),

there was an apparent rightward shift in the peak PNMT mRNA fraction when intact and ACTH-treated intact animals were compared to hypophysectomized and ACTH-treated hypophysectomized animals. The rightward shift suggested that fewer ribosomes were associated with each mRNA molecule, indicating a reduction in the usage of the messenger RNA for protein translation. However, the shift in peak position was only one fraction, and more likely reflects slight differences in fraction collection or gradient formation.

When the relative magnitudes of the PNMT mRNA peaks were compared, half as much PNMT mRNA was utilized for protein synthesis by the hypophysectomized rats, whereas four and five times as much PNMT mRNA was utilized for protein synthesis by the ACTH-treated intact rats and ACTH-treated hypophysectomized rats respectively. Furthermore, in the latter case, the incremental utilization of mRNA was equivalent to the absolute rise in PNMT mRNA in the total RNA pool, indicating that the entire pool of PNMT mRNA was used for protein production.

When the ribosomal loading pattern was examined for the chronic ACTH paradigm, there were some notable differences (Figure 6). Examination of the adrenal polysome distribution showed that hypophysectomy resulted in a rightward shift of the larger polysomal fractions without a concomitant shift in the monosome/disome fractions. Hence, there was an apparent decline in the number of ribosomes loaded onto each molecule of mRNA and a reduction in protein translation from the entire mRNA pool. However, long-term ACTH treatment appeared to reverse the effects of hypophysectomy and glucocorticoid depletion, because the polysomal distribution profile for the ACTH-treated hypophysectomized animals was very similar to that observed for the intact animals.

The polysome fractions were further examined as described previously for PNMT mRNA content (Figure 7). No shift in the PNMT mRNA peak fraction was observed, suggesting that no change in the utilization of PNMT mRNA for translation occurred for any of the treatment groups. However, comparison of the magnitude of the PNMT mRNA peaks indicated that ~1.5-fold more PNMT mRNA served as template for protein synthesis in the hypophysectomized rats. Given that the total available adrenal PNMT mRNA pool in hypophysectomized rats and chronically ACTH-treated hypophysectomized rats was equivalent and 50% of that observed for intact rats respectively, it would appear that under normal circumstances a portion of the PNMT mRNA pool may be held in reserve and not utilized for protein translation.

Thus, the results from the ribosomal loading studies indicate that the pools of available PNMT mRNA are fully utilized for translation in response to an acute elevation in endogenous glucocorticoids and likely only partially utilized in response to chronic glucocorticoid treatment. However, in response to an acute increment in glucocorticoids, fewer ribosomes may be loaded onto each PNMT mRNA molecule leading to a reduction in the efficiency of utilization of RNA template for PNMT protein synthesis.

DISCUSSION

In the preceding studies, we demonstrate several novel findings with respect to glucocorticoid regulation of adrenal medullary PNMT expression, which may have important implications for PNMT and epinephrine expression in response to acute and chronic changes in glucocorticoids, as in the case of stress. First, the mechanisms controlling PNMT expression appear to be different in response to acute and chronic glucocorticoid replacement therapy. Second, glucocorticoid-induced changes in PNMT gene transcription occur much more rapidly than heretofore reported. Third, PNMT gene transcription and protein expression are apparently uncoupled, with glucocorticoids exerting independent effects on these intracellular events. Lastly, the final common pathway for corticosteroid control depends on the ultimate expression of enzymatically active PNMT, as the amount of functional enzyme determines the production of epinephrine.

Previous studies have, for the most part, examined the response of PNMT to chronic glucocorticoid changes as changes in PNMT enzymatic activity appeared to require sustained corticosteroid exposure (Ciaranello et al. 1975). However, we have recently demonstrated that PNMT mRNA responds very rapidly to either neural or hormonal stimuli (Wong et al. 1992; Wong et al. 1993), prompting us to examine the effects of both acute and chronic glucocorticoid manipulation on PNMT mRNA and enzyme activity. On so doing, it became apparent that acute and chronic mechanisms controlling PNMT expression differed, and that in both cases, PNMT gene transcription and protein expression were uncoupled.

Much evidence has accumulated supporting the hypothesis that glucocorticoid control of gene transcription constitutes a major regulatory mechanism governing PNMT expression. For example, dexamethasone or corticosterone has induced PNMT mRNA levels in hypophysectomized and intact rats, cultured rat adrenal medulla, and bovine chromaffin cells (Jiang et al. 1989; Wan and Livett 1989; Ross et al. 1990; Evinger et al. 1992; Wong et al. 1993). In vivo ACTH also mimics the effects of corticosteroids (Evinger et al. 1992), presumably by stimulating the production of endogenous corticosterone. It appears that the rise in PNMT mRNA is due to an increase in the rate of gene transcription and further, that the entire pool of PNMT mRNA is functional for protein synthesis (Evinger et al. 1992).

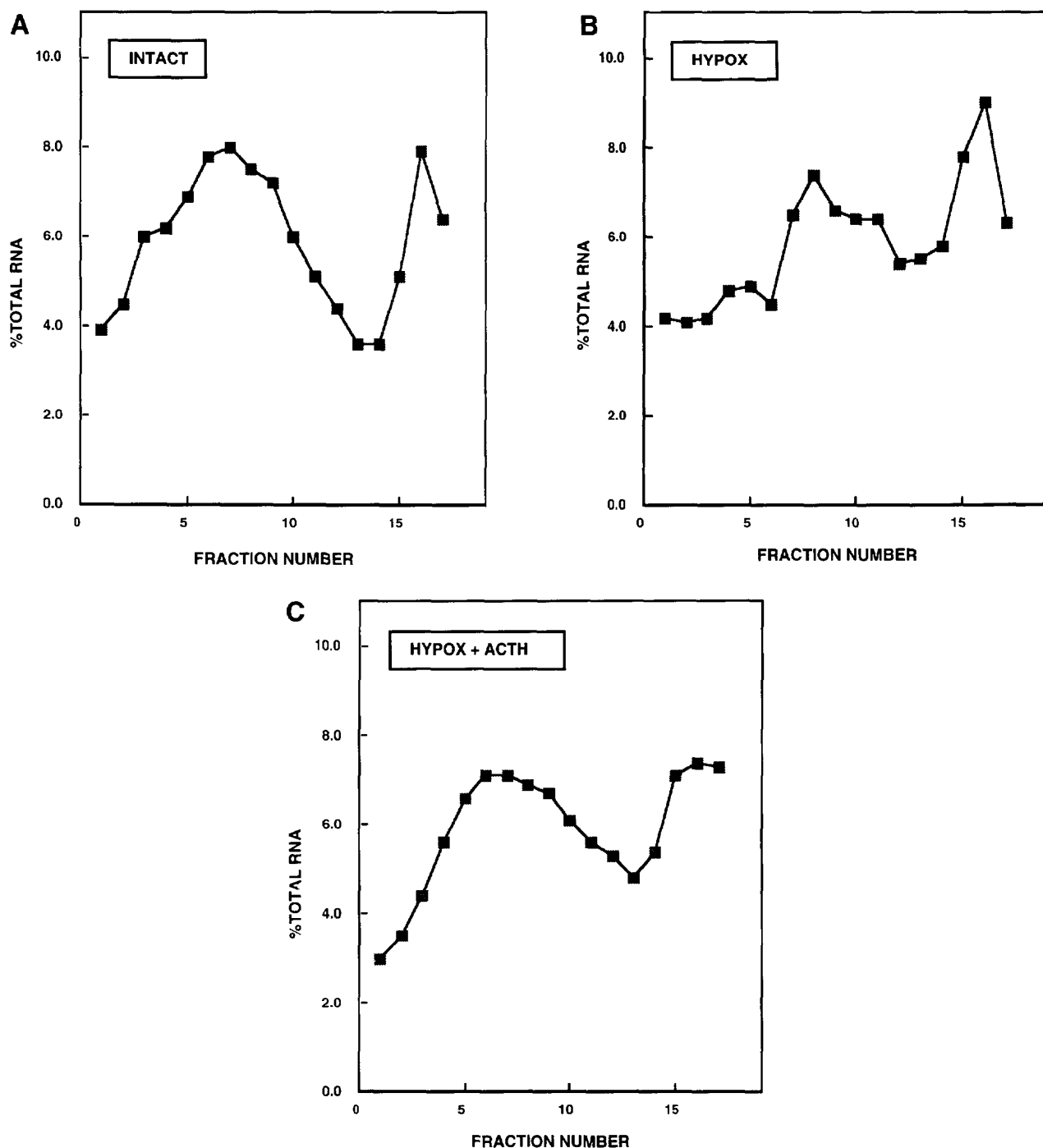In contrast to earlier reports, we did not observe

Figure 6. Polysome distribution, chronic ACTH treatment. Hypophysectomized rats were treated with ACTH (4 IU SC daily for 7 days) and killed 24 hours after the final injection. Controls included vehicle-injected intact and hypophysectomized animals. Polysomes, prepared from the adrenal glands, were separated on a linear sucrose gradient (0.5 to 1.5 M) as described in the Materials and Methods section. Fractions of 0.6 ml were collected and the A_{260} determined for each to generate the polysome distribution profiles shown. (**A**), intact; (**B**), hypophysectomized; and (**C**), ACTH-treated hypophysectomized.

a decline in adrenal PNMT mRNA after hypophysectomy, as might be expected if glucocorticoids were required to maintain PNMT gene transcription, although PNMT enzymatic activity was markedly reduced (Ciaranello et al. 1975; Wong et al. 1982; Wong et al. 1985; Jiang et al. 1989; Evinger et al. 1992). The difference may, in part, be due to experimental design. In our studies, drug treatment was initiated at 10 days
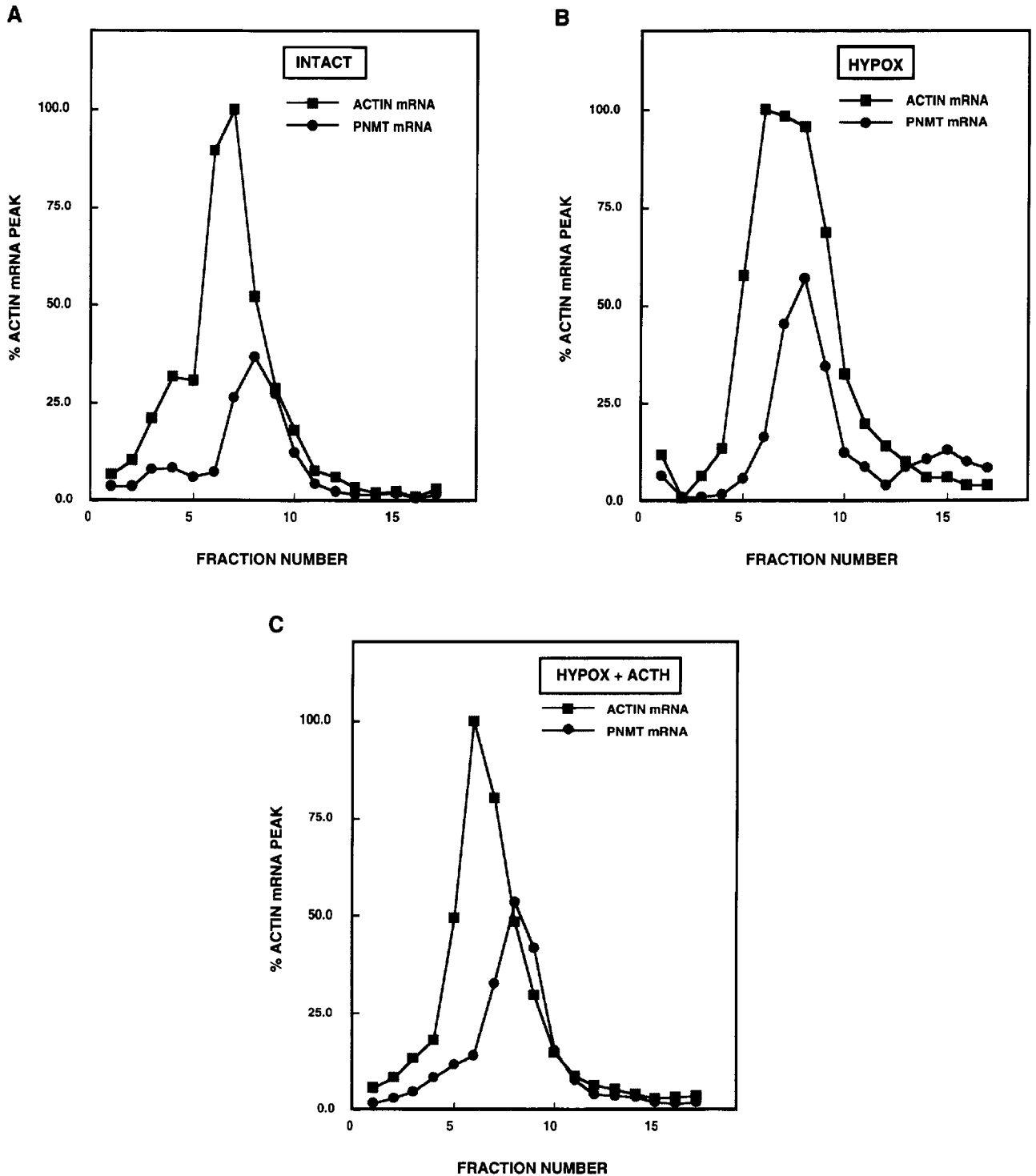
Figure 7.   Utilization of PNMT mRNA pool, chronic ACTH treatment. Each polysome fraction shown in Figure 6 was analyzed for PNMT by RNase protection assay as described in the Materials and Methods section. mRNA levels in each fraction are expressed relative to the peak β-actin fraction (100%) for the treatment group. (**A**), intact; (**B**), hypophysectomized; and (**C**), ACTH-treated hypophysectomized.

posthypophysectomy, rather than at 2 to 3 weeks, because we have found that PNMT restoration becomes more difficult and less reliable if delayed for longer than 10 days after surgery (Ciaranello et al. 1975). Using this

timetable, sufficient PNMT mRNA was present in 3 μg of total adrenal RNA to be well within the detection limits for either RNase protection assays (Wong et al. 1993) or Northern analysis (Wong et al. 1992). In addi-

tion, although many investigators have interchangeably studied PNMT regulation in vivo in rats, in cultured rat adrenal medulla or in cultured bovine chromaffin cells and the rat and bovine PNMT genes seem to share many common transcriptional regulatory elements, there is a growing concern that important species-specific differences exist. Finally, although glucocorticoid and ACTH paradigms both elevate glucocorticoid levels, they differ in one very critical respect. Glucocorticoid treatment sustains corticosterone concentrations beyond a normal physiologic level, whereas ACTH treatment, which stimulates endogenous corticosterone production, is subject to the normal physiologic autoregulatory events governing glucocorticoid and GR concentrations (Kalinyak et al. 1987).

Consistent with glucocorticoid control of PNMT gene expression, it has previously been reported that acute and chronic exposure to high cortisol increases the amount of nuclear, activated glucocorticoid receptor, and that a 1.5-fold to 2.0-fold rise in PNMT activity subsequently follows (Betito et al. 1992). As further evidence, the present studies show that GR mRNA expression is elevated at the peak of PNMT mRNA expression in the acute ACTH paradigm. However, the pattern of GR mRNA changes seen in the acute ACTH paradigm differed somewhat from anticipated. GR mRNA rose three-fold after hypophysectomy and then declined when ACTH treatment was initiated. In vivo, GR mRNA expression is autoregulated (Kalinyak et al. 1987; Dong et al. 1988), so that in response to dexamethasone, its levels decline. Corticosteroid depletion may therefore have the opposite effect, that of inducing GR mRNA expression, and further, when corticosterone levels rise in response to ACTH, GR mRNA then declines.

To determine why changes in PNMT mRNA were not reflected by changes in PNMT enzymatic activity, ribosomal loading experiments were executed to see whether PNMT mRNA utilization for protein translation differed. PNMT mRNA was fully utilized in the acute ACTH paradigm where mRNA was elevated, while PNMT activity remained suppressed. In contrast, PNMT mRNA was only partially utilized in the chronic ACTH paradigm under conditions where PNMT mRNA was 50% of normal, while PNMT activity had been restored. It is clear that differences in protein translation alone are insufficient to explain why changes in active protein and mRNA do not correspond. However, glucocorticoids have also been demonstrated to control PNMT proteolysis (Ciaranello 1978; Berenbeim et al. 1979; Wong et al. 1982; Wong et al. 1985). After corticosteroid depletion, proteolytic degradation of PNMT increases, apparently due to a depletion in the cosubstrate and methyl donor, SAM. Glucocorticoids appear to sustain the SAM metabolic enzymes, S-adenosyl-homocysteine hydrolase and methionine adenosyl-transferase (Wong et al. 1985), which, in turn, sustain

SAM concentrations. When SAM is bound to PNMT, it masks a proteolytic site(s), reducing the vulnerability of PNMT to degradation. Hence, if the rate of protein synthesis does not change while the rate of degradation decreases, then the net effect is an increase in functional enzyme. Moreover, it is this final level of glucocorticoid control that governs the ultimate expression of functionally active PNMT enzyme upon which the biosynthesis of epinephrine depends.

Thus, although it could be argued that glucocorticoids play a major role in PNMT gene expression, their most critical effects occur posttranscriptionally and, apparently, posttranslationally through the control of PNMT degradation and the amount of enzymatically active protein.

ACKNOWLEDGMENTS

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