Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 7261–7265, October 1987
Medical Sciences

Characterization of proopiomelanocortin transcripts in human nonpituitary tissues

(testis/RNA blot hybridization/S1 nuclease mapping/transcription)

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Communicated by Alfred Jost, June 1, 1987 (received for review February 23, 1987)

ABSTRACT **Proopiomelanocortin (POMC), the precursor to adrenocorticotropic hormone and other related peptides, was originally identified in the corticotropic cell. Recent evidence shows that POMC products are also normally present in a variety of nonpituitary tissues. To investigate this phenomenon in humans we looked for the presence and characteristics of POMC transcripts in various adult tissues. Blot hybridization analysis of normal adrenal, thymus, and testis RNAs revealed a small RNA species approximately 400 nucleotides shorter than the 1200-nucleotide pituitary species. Primer extension and S1 nuclease mapping studies showed that this small RNA lacked exon 1 and exon 2 of the gene, and it corresponded to a set of at least six molecules starting 41 to 162 nucleotides downstream from the 5′ end of exon 3. These RNAs appear to result from heterogeneous transcription initiation sites presumably under the control of "GC box" promoter sequences located in the 3′ end of intron 2. They cannot encode a complete POMC molecule, and the only truncated POMC molecules that could be translated would lack a signal peptide necessary for membrane translocation and precursor processing. The use of highly sensitive S1 nuclease mapping techniques with uniformly labeled single-stranded DNA probes allowed the detection of a small but definite amount of the "normal," 1200-nucleotide, mRNA species. It is suggested that it is this POMC mRNA that is responsible for the local production of all the POMC peptides.**

Proopiomelanocortin (POMC) is a polypeptide molecule that was identified as a precursor to adrenocorticotropic hormone (ACTH) in corticotropic cells (1, §). Its primary structure was established after the cDNA sequence was obtained from beef pituitary mRNA (2), and the gene structure was unravelled in many species, including humans (3–6). These data provided the molecular basis for the mechanism of POMC processing which, besides ACTH, releases a number of fragments with potential biological activity such as the 16-kDa fragment, the melanocyte-stimulating hormones, the lipotropins, and the endorphins (7).

Numerous reports have recently shown that POMC peptides are also normally present in extracts of various nonpituitary tissues including brain, gastrointestinal tract, placenta, and male and female gonads (7). One of the most extensively studied tissues is the testis. Immunocytochemical studies with antisera directed against the γ3-melanocyte-stimulating hormone, ACTH, and β-endorphin of the precursor have located immunostainable POMC products in Leydig cells of many species (8). The molecular forms of these products, as assessed by gel exclusion and HPLC techniques, indicated that POMC processing in the rat testis is similar to that in the pituitary intermediate lobe cell (9).

POMC mRNA was subsequently identified in rat and mouse testis by blot hybridization analysis (10, 11) and precisely located in the mouse Leydig cell by *in situ* hybridization (12), providing the best evidence for POMC gene expression in this tissue. Surprisingly, the POMC transcripts were quite smaller than those in the pituitary (11), and the peptide content appeared extremely low for the amount of POMC mRNA in comparison to what is observed in other tissues (9).

In this report we have identified and characterized the POMC transcripts in normal human nonpituitary tissues, mainly in the testis. Most of these transcripts are truncated molecules lacking the 5′ end of the coding region. On these grounds we propose an explanation for the small amount of all the POMC products, which are nevertheless present in these tissues.

MATERIALS AND METHODS

Isolation of RNA. Normal human pituitaries and nonpituitary tissues were obtained at autopsy or as a by-product of surgery. Total RNA was extracted by the guanidium chloride procedure (13) as modified in our laboratory (14). Poly(A)-enriched fractions were isolated by oligo(dT)-cellulose chromatography (15), with a yield of about 5% (wt/wt).

Blot Hybridization Analysis. RNA samples were denatured with 10 mM methylmercuric hydroxide (16), fractionated on denaturing 2% agarose gels, blotted onto nylon filters (GeneScreen*Plus* from New England Nuclear), and hybridized with single-stranded DNA probes prepared from phage M13 subclones containing POMC genomic fragments and encompassing each of the three exons of the human gene.

Probe Synthesis. Single-stranded DNA probes of a defined size, complementary to POMC genomic fragments subcloned in M13 vector, were obtained by extension from the M13 universal primer (17). Two probes were complementary to the coding region of the third exon: pHOX 3A, 707 bases (b) long, encompassed 657 b of the gene; pHOX 3C, 155 b long, was generated by extension from the 5′-CCCGCTGAGAC-GTCCTC-3′ oligonucleotide complementary to the third exon and included 8 b of vector sequence at its 3′ end. This POMC-specific primer was also labeled with polynucleotide kinase to generate a 5′-end-labeled probe.

S1 Nuclease Mapping. The technique was performed as described previously (18). Briefly, uniformly or 5′-end-la-

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Abbreviations: ACTH, adrenocorticotropic hormone; POMC, proopiomelanocortin; b, base(s); U, units.

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§Orth, D. N., Nicholson, W. E., Shapiro, M. & Byyny, R., Program of the Endocrine Society, 52nd Meeting, June 1970, St. Louis, p. 140 (abstr.).

beled single-stranded probes were hybridized to RNA in 10 μl of 40 mM 1,4-piperazinediethanesulfonic acid (Pipes), pH 6.4/80% (vol/vol) formamide/0.4 M NaCl at 48°C (56°C with pHOX 3A probe) for 12 hr. Then the heteroduplexes were digested in 200 μl of 250 mM NaCl/50 mM sodium acetate, pH 4.6/2.5 mM $ZnSO_4$ containing S1 nuclease at 5000 units (U) (as defined by the supplier, Bethesda Research Laboratories) per ml for 2 hr at 25°C. After extraction with phenol and precipitation with ethanol, protected fragments were separated on a 6% (4% for pHOX 3A-generated fragments) polyacrylamide/7 M urea gel.

Primer Extension. RNA samples were first denatured 20 min in 90% (vol/vol) dimethyl sulfoxide at 45°C and precipitated with ethanol. Then they were hybridized to the 5'-labeled POMC primer in 10 μl of 100 mM Tris·HCl, pH 7.5/150 mM KCl/60 mM $MgCl_2$ at 65°C for 15 min and at 42°C for 1 hr. Each sample was adjusted to 20 μl of 50 mM Tris·HCl, pH 7.5/75 mM KCl/3 mM $MgCl_2$/2 mM sodium pyrophosphate/1 mM dNTP with 40 U (as defined by the supplier) of the Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) and extended for 1 hr at 42°C. After extraction with phenol and precipitation with ethanol, the extension products were separated on a 6% polyacrylamide/7 M urea gel.

RESULTS

Identification of the Small POMC Transcripts in Human Tissues. Total or poly(A)$^+$ RNA prepared from normal adrenal, thymus, testis, and pituitary was subjected to blot hybridization analysis. The hybridization was performed with a 707-b single-stranded DNA probe encompassing most of the third exon coding region (pHOX 3A). POMC transcripts were detected in the testis and thymus (Fig. 1) and adrenal glands (not shown); they were polyadenylylated and their size (≈800 b) was approximately 400 b shorter than that of pituitary POMC mRNA. The intensity of the signal indicated that POMC transcripts were more abundant in the testis than in the adrenal and thymus. No hybridizable material could be detected, on the same filter, with probes complementary to the first or second exon of the gene.

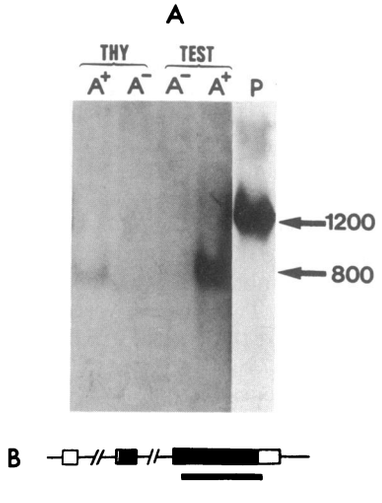

FIG. 1. Blot hybridization analysis of testis, thymus, and pituitary RNA. (*A*) Thymus (THY) and testis (TEST) poly(A)$^+$ RNA (5 μg, A$^+$) and poly(A)$^-$ RNA (20 μg, A$^-$) and total pituitary RNA (0.5 μg, P) were denatured in 10 mM methylmercuric hydroxide and separated on a denaturing 2% agarose gel. The RNAs were blotted onto a GeneScreen*Plus* sheet and hybridized with the uniformly labeled pHOX 3A probe. The sheet was exposed 3 days at −80°C. Lengths are given in b. (*B*) Human POMC gene structure. Exons are indicated as boxes, the stippled part corresponding to the protein-encoding regions. The solid bar represents the pHOX 3A probe.

Characterization of the Small POMC Transcripts by S1 Nuclease Mapping and Primer Extension Studies. Uniformly labeled pHOX 3A probe was hybridized with testis poly(A)$^+$ RNA and subjected to S1 nuclease digestion, and the protected fragments were separated by electrophoresis (Fig. 2). Several bands were detected. The slowest one was 657 b long, corresponding to the entire POMC-specific part of the probe, and was similarly protected in the pituitary (Fig. 2, lane 3); smaller fragments, at least three, were also detected, with a size of approximately 600 b. To better characterize these protected fragments we used a smaller, 155-b single-stranded DNA probe (pHOX 3C) generated from a synthetic 17-mer primer complementary to the position 7013–7029 fragment of the gene (Fig. 3*C*). S1 nuclease mapping studies were performed on testis, thymus, and pituitary RNA, using first a uniformly labeled pHOX 3C probe (Fig. 3*A*); five protected fragments were detected. The slowest one was 147 b long, corresponding to the entire POMC-specific part of the probe, and was similarly protected in the pituitary (Fig. 3*A*, lane 4). The four others were specific for the nonpituitary tissues and were more abundant in the testis than in the thymus (Fig. 3*A*, lane 5). When the same studies were performed with the 5'-end-labeled pHOX 3C probe, the same protected bands were again observed (data not shown), confirming that the heterogeneity of these POMC transcripts resulted from variable 5' extremities. In this latter experiment, similar intensities of the signals also indicated that each POMC transcript was present in approximately equal amounts.

To precisely locate the 5' end of the transcript protecting all the POMC sequence of pHOX 3C, primer extension studies were performed with the synthetic 17-mer primer (Fig. 3*B*). The longest extension product was 157 b long, indicating a transcript with a 5' end starting 13 b upstream from the *Sma* I 5' cloning site of pHOX 3A and pHOX 3C probes, that is 41 b downstream from the 5' end of exon 3. A smaller, 36-b, fragment was also observed, which probably corresponded to yet another, and shorter, POMC transcript that had not been detected by the S1 mapping studies; it might be explained either by a poor stability of this short heteroduplex under the stringent hybridization conditions or by an artifactual arrest of the extension reaction, which can also account for some additional intermediary bands.

Since prior S1 nuclease mapping studies directed at the 3' end of exon 3 had provided identical results with testis or pituitary RNA (data not shown), it could be concluded that the heterogeneity of the testis POMC transcripts resulted entirely from their variable 5' extremities. Thus, the combination of S1 mapping and primer extension studies allowed

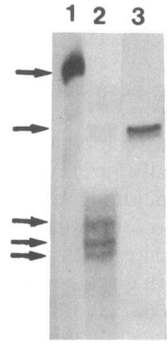

FIG. 2. S1 nuclease mapping of the 5' end of testis small POMC transcripts. Testis poly(A)$^+$ RNA (10 μg, lane 2) and pituitary total RNA (0.5 μg, lane 3) were hybridized with probe pHOX 3A at 56°C for 12 hr, and the products of S1 nuclease digestion (5000 U/ml) were separated on a 7 M urea/4% polyacrylamide gel. The untreated probe was in lane 1.

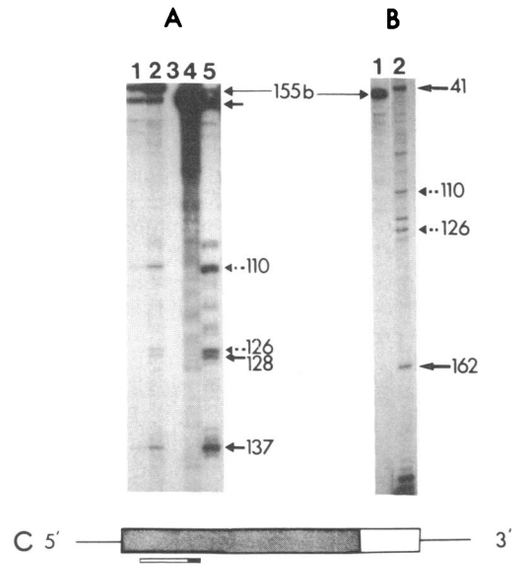

FIG. 3. S1 nuclease mapping and primer extension studies of the 5′ extremities of the small POMC transcripts. (*A*) Total or poly(A)$^+$ RNA was hybridized with the pHOX 3C probe at 48°C for 12 hr, and the products of S1 nuclease digestion (5000 U/ml) were separated on a 7 M urea/6% polyacrylamide gel. Lane 1, 10 μg of thymus poly(A)$^+$ RNA; lane 2, 15 μg of testis poly(A)$^+$ RNA; lane 3, pHOX 3C probe treated without RNA; lane 4, 0.5 μg of pituitary total RNA; lane 5, 50 μg of testis total RNA. (*B*) Testis poly(A)$^+$ RNA (5 μg, lane 2) was hybridized with the 5′-end-labeled primer, and extension products were analyzed on a 7 M urea/6% polyacrylamide gel together with pHOX 3C probe (lane 1). In *A* and *B*, dashed arrows point to the initiation sites similarly identified by the two methods. Numbers indicate the positions relative to the 5′ end of exon 3; subtracting them from the length of exon 3 (833 b) indicates the sizes of the various transcripts. (*C*) Schematic representation of human POMC gene exon 3. The 155-b pHOX 3C probe is indicated by the thin bar below, with the 17-mer synthetic primer in black.

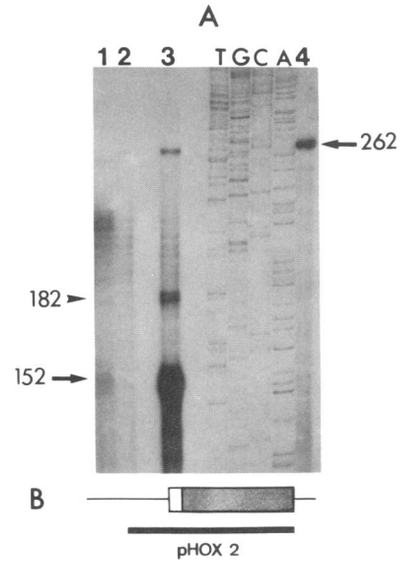

FIG. 4. S1 nuclease mapping of POMC exon 2 with pituitary and testis RNA. (*A*) Testis total RNA (50 μg, lane 1), lung poly(A)$^+$ RNA (50 μg, lane 2), and pituitary total RNA (0.5 μg, lane 3), were hybridized with the pHOX 2 probe at 48°C for 12 hr, and the products of S1 nuclease digestion (5000 U/ml) were separated as for Fig. 3. The untreated probe was on the same gel (lane 4). Sanger sequencing tracks (T,G, C, and A) were used for molecular weight markers. (*B*) Human POMC gene structure. The 262-b pHOX 2 probe is indicated by the solid bar under exon 2.

the complete characterization of six predominant POMC mRNA species in the human testis: their variable sizes of 793, 724, 708, 706, 697, and 672 b [without the poly(A) tail], corresponding to a variable 5′ end starting 41, 110, 126, 128, 137, and 162 b, respectively, downstream from the 5′ end of exon 3 (see Fig. 5).

Detection of the 1200-b POMC mRNA in Human Testis by S1 Nuclease Mapping Studies. S1 nuclease mapping experiments were also performed with uniformly labeled single-stranded DNA probes encompassing exon 2 (pHOX 2, Fig. 4) and exon 1 (data not shown). This highly sensitive method allowed the detection of "normal" (1200-b) POMC mRNA in the testis, which had not been observed by blot hybridization analysis even after a prolonged exposure. As shown in lane 3 of Fig. 4, a 152-b fragment was indeed protected by testis RNA; this fragment corresponded to the entire POMC exon 2, and it was similarly protected in the pituitary (Fig. 4, lane 1). The second, 182-b, fragment protected by pituitary RNA (lane 1) corresponds to an alternative splice site of the first intron of the gene and will not be discussed in this paper. Thus, although the 800-b species appears to be predominant, a small but definite amount of the 1200-b POMC mRNA could also be demonstrated in human testis.

DISCUSSION

This report demonstrates the presence of POMC transcripts in various nonpituitary human tissues. Because blot hybridization analysis revealed their peculiar size, approximately 400 b shorter than the "normal" 1200-b pituitary mRNA, it was necessary to further characterize these molecules. This could be done best with the testis, which provided a substantial amount of POMC transcripts.

The uniformly labeled single-stranded DNA probes proved to be a most efficient tool to perform highly sensitive S1 nuclease mapping studies. Together with primer extension, this technique characterized the transcripts as a population of at least six different RNAs with heterogeneous 5′ extremities starting 41 to 162 b downstream from the 5′ end of exon 3. This result raised the question of the mechanism responsible for this tissue-specific size polymorphism.

Since there is only one POMC gene per human haploid genome (19), this phenomenon could only result either from a variable splicing event or an alternative mode of transcription initiation. The first hypothesis is doubtful, since no 3′ consensus site (20) for an intron is present in the 5′ region of exon 3. The second hypothesis therefore appears more likely. It is supported by the recently described mechanisms of transcription initiation in a peculiar class of genes called "housekeeping genes," such as those encoding hydroxymethylglutaryl-CoA reductase (21) and dihydrofolate reductase (22). The promoter sequences of these genes lack a typical "TATA box" but contain G+C-rich regions and some typical repeats of the sequence

$$\begin{matrix} TA & & TAAT \\ GGGGCG & & GGGC \end{matrix}$$

("GC box"), which induce heterogeneous initiation of transcription, greatly stimulated after binding with the ubiquitous transcriptional factor Sp1 (23). In this regard it is remarkable that the 5′ flanking region of the short POMC transcripts also corresponds to a G+C-rich region of the gene and does contain two typical GC boxes and several related sequences (Fig. 5). We suggest that the small POMC transcripts that are found in many (but not all) nonpituitary tissues result from an alternate mode of POMC gene transcription using a GC box promoter sequence. This mode of POMC gene expression could be turned on by nonspecific factors such as Sp1 when chromatin structure and DNA methylation pattern are compatible with a transcriptional activity. It is generally accepted that transcription of a

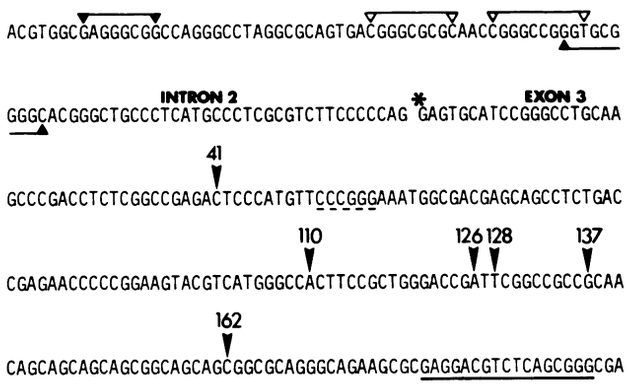

FIG. 5. Nucleotide sequence of the intron 2–exon 3 region of the human POMC gene. The multiple transcription initiation sites of the small POMC transcripts (▼), the GC box (▼▼) and related sequences (▽▽), the 17-mer primer (——), the 5′ end of exon 3 (*), and the *Sma* I cloning site (----) are indicated. Numbers indicate base position relative to the 5′ end of exon 3.

given gene requires that its chromatin environment be in a relaxed state, a condition that can be detected by an increased sensitivity to DNase digestion (24). If such a state of relaxed chromatin environment were "actively" obtained for the human POMC gene in nonpituitary tissues it would suggest that the small (800-b) transcripts might have some, yet unknown, biological function; this function might depend either on the RNA molecules themselves or on their possible translation products derived from the POMC or the non-POMC reading frames. Alternatively, the relaxed chromatin state of the gene might be a "passive" phenomenon: a low level of gene expression can be reminiscent of a higher activity during fetal development, as observed, for example, for the α-fetoprotein gene (25). Another hypothesis would be that the human POMC gene is located in the vicinity of actively transcribed genes, which induce chromatin conformation changes that encompass the POMC gene. This phenomenon of neighbor gene transcriptional activation is well documented; it is responsible for the tissue-specific activation of various oncogenes such as c-*myc* in Burkitt lymphoma (26). Whatever the mechanism of this related chromatin conformation may be, the use of the "normal" (pituitary) promoter sequence, which generates the 1200-b POMC mRNA, would require, in addition, cell-specific transactivating factors that must be present mainly in the pituitary corticotropic cell (27), and presumably in the hypothalamus (28) and some ACTH-producing nonpituitary tumors (27).

Another major question raised by these results concerns the putative translation products of the truncated POMC transcripts. None of them can code for a complete POMC molecule, since its normal initiation codon is located in exon 2. Analysis of the POMC reading frame of the small transcripts reveals two AUG codons that, according to Kozak's rule (29), could initiate their translation. The first one, present only in the longest transcript, 793 b, would initiate the translation of a 183-amino acid protein that lacks the NH_2-terminal part of γ3-melanocyte-stimulating hormone and the 16-kDa fragment. The second one would generate a 45-amino acid peptide composed of the COOH-terminal part of γ-lipotropin and all of β-endorphin. In both cases, the first amino acids are mostly hydrophilic and cannot constitute a valid signal peptide, therefore preventing the peptide translocation into the membrane compartment of the secretory pathway (30). In these conditions, it is unlikely that these truncated POMC peptides can undergo an adequate post-translational processing. Thus the question can be asked whether the POMC peptides found in the testis actually derive from these small POMC transcripts.

An approach to this problem is provided by the observation of two different nonpituitary tissues of the rat, the testis and the hypothalamus. Whereas the concentrations of the overall POMC transcripts are identical in the two tissues, the concentration of POMC peptides is 3 orders of magnitude lower in the testis. Since blot hybridization analysis showed that hypothalamus transcripts are mainly of the "normal" (pituitary) size (10), these data strongly suggest that the small POMC transcripts indeed have a very low, if any, functional activity, at least in terms of POMC peptide production.

For this reason it was logical to investigate the hypothesis that "normal" (1200-b) POMC transcripts were present in the testis, although probably in very low concentrations. This search was made possible through the sensitive technique of S1 nuclease mapping with uniformly labeled single-stranded DNA probes. This approach allowed the demonstration that such transcripts were indeed present in the testis, in a concentration much lower than that of the small transcripts. The existence of "normal" POMC mRNA could easily explain the production of all the POMC peptides in this tissue; the local function of these peptides remains to be assessed.

The authors thank J. Shine for his generous gift of the POMC DNA clones, Dr. B. Debré for collecting the human tissues, and Mrs. M. Le Scouarnec for her excellent secretarial assistance. The study was supported by Contrat de Recherche Externe 864003 from the Institut National de la Santé et de la Recherche Médicale.

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