Melanin-concentrating hormone (MCH) mRNA expression is induced by nerve growth factor and lithium in PC12 cells, whereas three large MCH RNA species are found in untreated cells. In this study, we investigated the structures, regulations of expression, and putative functions of these transcripts. Northern blot, rapid amplification of cDNA ends-polymerase chain reaction, reverse transcriptase-polymerase chain reaction, and sequencing experiments demonstrated that they are antisense RNAs complementary to the MCH gene. Two classes of antisense RNAs could be discriminated as follows: 1) non-coding unspliced RNAs that overlap mainly the coding part of the MCH gene; 2) spliced variant mRNAs complementary to the 3′-flanking end of the MCH gene and that encode putative proteins containing DNA/RNA binding domains. We named this new transcriptional unit AROM for antisense-RNA-overlapping-MCH gene. Spliced variant AROM mRNAs are expressed in a broad range of rat organs. Western blot and immunohistochemistry experiments revealed several proteins with cytoplasmic but also nuclear localization in PC12 cells. Time course studies during nerve growth factor and lithium treatment of PC12 cells indicated a reciprocal regulation of the MCH and AROM gene transcripts, reflected also at the level of AROM proteins. The major translational product is a 64-kDa protein (AROM-p64). Recombinant AROM-p64 displayed high binding to single-stranded DNA and poly(A) homopolymers suggesting that this protein could play a role in mRNA maturation/metabolism.

The AROM Gene, Spliced mRNAs Encoding New DNA/RNA-binding Proteins Are Transcribed from the Opposite Strand of the Melanin-concentrating Hormone Gene in Mammals*

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Melanin-concentrating hormone (MCH) is a cyclic peptide, first isolated from salmon pituitary glands on the basis of its skin paling property (1), then purified from rat hypothalami, and sequenced (2). Synthesis of MCH was localized almost exclusively in a large population of neurons present in the zona incerta and lateral hypothalamus area of the mammalian brain (3–5). The MCH axonal network projected widely throughout the brain and posterior pituitary but innervated poorly the external part of the median eminence in resting animals (5). This particular cellular and axonal distribution argued in favor of a neurotransmitter/neuromodulator role for MCH in a large spectrum of functions in the central nervous system (reviewed in Refs. 6 and 7). Recently, several laboratories have demonstrated changes in different behaviors following intracerebroventricular injection of MCH in the rat brain. In particular, MCH plays a particularly important role in the regulation of feeding behavior and associated pathologies such as obesity (8–11). Given the important biological functions of MCH, it is of great interest to define the mechanisms that regulate the expression of its cognate gene.

A single MCH-encoding gene has been identified in both rat and mouse (12, 13), and two distinct MCH gene systems have been found in primates (14, 15). The regulation of MCH gene expression has been most extensively investigated with in vivo models (reviewed in Ref. 7). However, we established recently that production of mature MCH mRNA (0.95 kb in length) can be induced in PC12 cells following treatment with nerve growth factor (NGF) and lithium (16, 17). Interestingly, we identified MCH RNA species of high molecular weight (1.4, 3.5, and 4.0 kb in length) in unstimulated PC12 cells; those transcripts transiently disappearing after a short term treatment with NGF and lithium. Because the increase in MCH mRNA content was not apparently associated with transcriptional activation, we hypothesized that some of the large MCH gene-related transcripts could be involved in the regulation of the processing or degradation of the MCH mRNA (16, 17).

In the present study, we described the isolation and characterization of the high molecular weight MCH RNAs in PC12 cells and in rat tissues. Interestingly, they are derived from a new gene located on the opposite DNA strand to the MCH gene, with extensive overlap existing between the 3′ end of this gene and the MCH transcriptional unit. Some of the antisense MCH RNAs display alternative splicing and encode new DNA/RNA-binding proteins, whereas other putative transcripts lack extensive open reading frames suggesting different functions for the two classes of antisense RNAs.
**EXPERIMENTAL PROCEDURES**

**Cell Culture**—PC12 cells were grown in Dulbecco’s minimal essential medium or RPMI 1640 medium supplemented with 10% heat-inactivated horse serum, 5% fetal calf serum, and 50 μg/ml gentamicin as described elsewhere (16). The cells were maintained in a humidified atmosphere at 37 °C and 5% CO₂. Cells were plated in 100-mm dishes and used at about 10⁷ cells/dish on the day of the experiments. Inducers were added to the culture medium at the final concentration of 50 nM NGF (laboratory-made preparation) and 20 mM LiCl. At the end of the treatments, the cells were washed twice with 1× phosphate-buffered saline (PBS) solution and directly used for RNA or protein extraction.

**Rat Tissues**—Adult (3 months; 180–250 g) male Wistar rats (Institut de Pharmacologie Moléculaire et Cellulaire-CNRS, Valbonne, France) were maintained in a 12-h light-dark cycle with food and water given ad libitum. Hypothalamus and different peripheral tissues (colon, jejunum, ileum, spleen, lung, liver, testis, thymus, kidney, muscle, heart, stomach, and hypophysis) were carefully dissected, immediately frozen on dry ice, and stored at −80 °C until required.

**Isolation of RNA and Northern Blot Analysis**—Total RNA was extracted by a phenol-guanidinium isothiocyanate method (18). RNA samples were electrophoresed through a 2.2 M NaOH solution and different tissues (colon, jejunum, ileum, spleen, lung, liver, testis, thymus, kidney, muscle, heart, stomach, and hypophysis) were carefully dissected, immediately frozen on dry ice, and stored at −80 °C until required.

**DNA Probes**—The rat MCH cDNA probe was pRMCH 11 cDNA (19). The rat tyrosine hydroxylase cDNA probe was a fragment of pT51 cDNA (20, 21). A probe was labeled with [γ-32P]dCTP and terminal transferase as described previously. DNA fragments covering the 5’- and 3’-flanking region and the coding part of the MCH gene (see Fig. 1A) were generated as described elsewhere (17) with primers listed in Table I. These probes were labeled with [α-32P]dCTP by the random-primer method using a commercial kit (Amplitube, France). The specific activity of the probe was 0.5–10⁹ dpm/μg. Oligonucleotide primers were labeled using [α-32P]dCTP and terminal transferase as described previously (16).

**Rapid Amplification of cDNA Ends (RACE)-PCR Experiments**—RACE reactions were performed according to the user manual of Marathon cDNA amplification kit (CLONTECH Laboratories) with 1 μg of total RNAs isolated from PC12 cells grown in Dulbecco’s minimal essential medium in the absence of NGF and lithium. Oligonucleotides primers were used for the first PCR were RMCH/1AP1 and RMCH/2DAP1 for identification of the antisense RNAs, respectively (see Table I). Composition of RACE-PCR buffer and added components were as described previously (15). The PCR product was 94 °C, 1 min, followed by 5 cycles, with 30 s at 94 °C, 4 min at 72 °C, then 5 cycles with 30 s at 94 °C, 4 min at 70 °C and finally 25 cycles with 20 s at 94 °C and 4 min at 68 °C. To improve specificity, secondary PCRs were performed using 5-μl aliquots of a 1:50 dilution of the primary PCR solution with oligonucleotides ARMCH/2AP2 and RMCH2/2AP2 for the 5’ and 3’ ends, respectively (see Fig. 3B), using the same PCR profiles as described above except that only 20 cycles were performed at the last step. Specific fragments were identified by Southern blotting analysis with genomic probes.

**RT-PCR Experiments**—Total RNAs from tissues were reverse-transcribed with oligo(dT) or the specific primer 9, and the subsequent PCRs were performed under conditions described elsewhere (16) with primers shown in Table I and Fig. 5A. The reaction products were analyzed on a 0.8% agarose gel, blotted onto nylon membrane (Hybond N, Amersham Pharmacia Biotech), probed with MCH genomic probes (see Fig. 1A), and autoradiographed.

**cDNA Cloning and Sequencing**—Bands of interest corresponding to RACE-PCR products were excised from 0.8% agarose gels, purified using Wizard PCR Peps DNA purification (Promega, France), and sub-cloned in pGEM-T easy vector (Promega). E. coli XL1 bacteria were transformed by high voltage electroporation (1500 V, 282 ohms, 40 microfarads) with a 2-μl aliquot of the ligation mixture. Plasmids from individual colonies were prepared and sequenced as described elsewhere (16).

**Preparation of Affinity Purified Antibodies**—Antisera were raised against glutathione S-transferase (GST)-AROM fusion proteins and prepared as described elsewhere (16). DNA sequences encoding rat AROM-p50 and AROM-p17 proteins were amplified and subcloned into the pGEX3X vector (Amersham Pharmacia Biotech) in frame with the sequence of GST. Constructs were checked by sequencing. Fusion proteins were expressed in E. coli strain BL21 (DE3) with isopropyl-1-thio-galactopyranoside for 4 h at 37 °C. After 10% SDS-polyacrylamide gel electrophoresis and copper staining, GST-AROM-p50 and GST-AROM-p17 fusion proteins were excised from acrylamide gels and lyophilized. New Zealand female rabbits were immunized with 400 μg of fusion protein in the presence of complete Freund’s adjuvant and boosted 1 month later with 200 μg of fusion protein previously transferred to Hybond C-extra nitrocellulose membranes (Amersham Pharmacia Biotech). Specific antibodies were recovered by a 2-min elution with 0.1 M glycine, 5.5% bovine serum albumin (BSA). Immediately after elution, purified antibodies were brought to pH 7.6 with 1 M Tris-Cl (pH 8.0), 0.5% BSA.

**Baculovirus Expression System**—A PCR product encoding mouse AROM-p64 was obtained by amplifying a cDNA clone (GeneBank™ accession number AA139022) and subcloned initially in the pGEM-T easy vector. A DNA fragment encoding rat AROM-p50 was purified after restriction of total RNA from RT-PCR experiments. BAC DNA was checked before the fragment encompassing AROM-p64 or AROM-p50 was introduced into a pVL1392 vector (PharMingen). pVL-AROM-p64 or pVL-AROM-p50 vector was co-transfected with linearized baculovirus DNA (BaculoGold, PharMingen) into Sf9 cells following the manufacturer’s recommendations. Supernatant containing recombinant viruses was harvested 72 h after transfection. Expression of AROM-p64 or AROM-p50 in infected Sf9 cells was checked by immunoblot analysis of total proteins as described below.

**Protein Preparation for Western Blot Analysis**—Baculovirus infected Sf9 cells were harvested 72 h after infection, washed with PBS solution, and lysed at 4 °C with a buffer consisting of 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.1% sodium deoxycholate, 1% Triton X-1, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM iodoacetamide. The cell suspension was homogenized and then centrifuged at 12,000 × g for 15 min at 4 °C. Pellet and supernatant were stored at −20 °C until use.

Total proteins extracts from PC12 cells were prepared after washing the cells twice with PBS × 1 and putting them in a lysis buffer (50 mM Hepes (pH 7.5), 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 10 ms sodium pyrophosphate, 1% phenylmethylsulfonyl fluoride, 1 mM aprotinin, 0.3% Nonidet P-40, 0.5 mM sodium orthovanadate, 0.5% sucrose, 0.5% Nonidet P-40, 0.5% Triton X-100). The suspension was then centrifuged at 12,000 × g for 10 min at 4 °C. Pellet and supernatant were stored at −20 °C until use.

**Immunoblotting**—Protein samples were denatured with 2% β-mercaptoethanol in Laemmli’s sample buffer, separated on a 10% SDS-PAGE, and transferred onto a Hybond C-extra membrane (Amersham Pharmacia Biotech). Non-fat dry milk (5% in TBS) was used as blocking agent (1 h at room temperature) and for antibody dilutions. Primary antibodies were incubated 16 h at 4 °C. Dilutions of the primary antibodies are indicated in the figure legends, and the secondary antibody (Immunotech Fab’/2) was used at 1:2500 dilution. Blots were washed after incubation with either primary or secondary antibodies, with a solution of TBS containing 0.1% Tween 20. Antibody complexes were revealed by chemiluminescence (ECL +, Amersham Pharmacia Biotech).

**Histochemistry**—PC12 cells (passage 16–17) were placed on coverslips precoated with 0.25 mg/ml polyllysine (Sigma) and grown for 24 h as described above. The cells were washed with PBS solution and fixed with 4% paraformaldehyde for 15 min at room temperature. The slides were incubated for 20 min in 0.1% Triton-X-100 (TBS solution) in 0.5% BSA. Then primary anti-serum was applied for 45–90 min at room temperature in 1× TBS containing 0.05% Triton X-100 (0.1% Ab, Ha 43 Ab a preimmune serum at 1/500 final; anti-mouse tyrosine hydroxylase (Chemicon) at 1/400 final). After two washings in TBS solution, anti-IgG from sheep or mouse coupled to peroxidase (ABC substrate; Sigma) was used to reveal
RESULTS

Identification of Antisense MCH RNAs in PC12 Cells—We determined first the degree of overlapping with the MCH gene of the large transcripts identified in PC12 cells. Identical Northern blots containing two RNA samples (lanes A and B; Fig. 1B) isolated from untreated control cells (C), or treated with NGF/lithium for 12 h (T12), were hybridized either with a MCH cDNA probe, different MCH PCR fragments covering the flanking regions of the MCH gene (probes A–D), or sense oligoprobes RPCR4 and RMCH6 (Fig. 1A).

Hybridization with 32P-labeled MCH cDNA probe confirmed the expression of large MCH transcripts of 4.0, 3.5, and 1.4 kb in unstimulated PC12 cells (C samples) and revealed the absence of the largest transcripts in 12 h-treated cells (T12 samples) (Fig. 1B, top right panel). As expected (16), the 0.95-kb band, which corresponds to mature MCH mRNA, was identified by using the MCH cDNA probe only in the NGF/lithium-treated PC12 cells (T12 samples). The 4.0-kb transcript was identified with genomic probes C and D indicating that this RNA overlaps essentially with the 3′- and 5′-flanking regions. However, probe A did not display hybridization to the 4.0-kb RNA in the C samples suggesting that it does not extend far upstream from the 5′ end of the MCH gene. Interestingly, the 3.5-kb RNA species was clearly detected with probes C and D but not with probes A and B suggesting that this transcript contains essentially sequences of the 3′-flanking region of the MCH gene. In sharp contrast, the 1.4-kb RNA species was visualized with probe B but not with probes A, C, and D indicating that this RNA overlaps essentially with the immediate 5′-flanking region of the MCH gene. Finally, hybridization with oligoprobes RPCR4 and RMCH6 revealed a pattern similar to this found with probe B, suggesting that at least the 4.0- and 1.4-kb RNA species must be transcribed in the opposite direction to that encoding the MCH mRNA. By using additional Northern blot analysis, antisense RNAs corresponding to the 1.4-kb transcripts were further demonstrated with single-stranded MCH RNA probe (data not shown).

Structural Analysis of the AROM Gene and Spliced Transcripts—We used a RACE (rapid amplification of cDNA ends) protocol to characterize the antisense MCH RNAs. By using primer RMCH2, we found only cDNAs ending with a stretch of dT residues in untreated PC12 cells, confirming the synthesis of antisense MCH mRNA (Fig. 2B). Further evidence that this was the true orientation was the presence of canonical poly(A) addition sites at the expected distances from the A regions in these RACE-PCR products (bracket in Fig. 3). In contrast, extension of the cDNAs using primers oriented in the opposite direction (including ARMCH2) produced numerous RACE products lacking a poly(dT). Bands corresponding to a broad range in length of RACE products were purified, subcloned into a PGE-T cloning vector, and sequenced (Fig. 2B). All the RACE products overlapped the coding part and large portions of the 3′-flanking region of the MCH gene but differed markedly in their 5′ end and exhibited internal heterogeneity. These observations indicate that a gene extending past the 3′-flanking region of the MCH gene but on the opposite DNA strand generates several transcripts through alternative promoter use or/and alternative splicing. We named this new gene AROM immunoreactivity and tyrosine hydroxylase immunoreactivity, respectively. Controls to ensure non-cross-reactivity of secondary antisera with inappropriate antigens were carried out and were negative.

DNA and RNA Binding Assays—DNA/RNA affinity chromatographies were performed essentially as described by Kadonaga (23). Briefly, baculovirus-infected SF9 cells that produced mouse AROM-p54 or rat AROM-p50 were lysed either with a solution containing 25 mM Hepes (pH 7.6), 100 mM KCl, 0.2 mM EDTA, 1 mM dithiothreitol, 20% glycerol, 0.1% Nonidet P-40, and complete inhibitor protease (Roche Molecular Biochemicals) (solution A) or in the same buffer where 12.5 mM MgCl2 replaced 0.2 mM EDTA (solution B). SF9 cell sonicate was centrifuged at 14,000 rpm for 10 min, and the pellet and supernatants were stored at −20 °C. Denatured calf thymus DNA-conjugated to agarose or cellulose beads (Amersham Pharmacia Biotech) was used for DNA binding reactions. Ribonucleotide polymers (poly(A) and poly(U) from Amersham Pharmacia Biotech and poly(C) and poly(G) from Sigma) were used for RNA binding reactions. 200 μl of pellet containing AROM proteins was incubated at 4 °C for 1–3 h with RNA- or DNA-conjugated agarose/cellulose-Sepharose beads. After a brief spin in a microcentrifuge, the beads were pelleted, and the supernatant (BT) was stored. The beads were washed four times with 150 μl of binding buffer (solution A or B) to remove unspecific protein binding. Generally, bound protein was eluted from DNA/RNA resins by successive washings with increased ionic strengths (150 μl of solution A or B containing from 0.2 to 1 M NaCl). Finally, the beads were washed once with 40 μl of the solution A or B and denatured with 2% 2-mercaptoethanol Laemmli’s sample buffer. Proteins were analyzed by Western blots as described below.

Fig. 1. Expression of the MCH gene-related transcripts in PC12 cells. A, schematic representation of the rat MCH gene. The three exons are boxed in black. Locations and lengths of the cDNA, oligoprobes, and genomic DNA probes are indicated. The Pet/Pet fragment has been fully sequenced (17). B, Northern blot analysis of MCH mRNA and high molecular weight RNAs in control PC12 cells (C) and 12 h NGF/lithium-treated cells (T12). Fifteen microgram samples (in duplicate; lanes A and B) of whole cell RNA were hybridized to 32P-labeled MCH genomic probes (A–D), MCH cDNA, or sense oligoprobes. The lengths of the MCH gene-related transcripts are indicated on the right. Mature MCH mRNA is noted by an arrowhead.

40578 Antisense MCH Gene Transcripts
for antisense-RNA-overlapping-MCH gene (Fig. 2A, GenBank™ accession number nkit 356009; AF303035).

We screened one gene database (GenBank™) and an EST data base (Merck-washed EST project). We identified EST sequences corresponding to the human (EST, AA251058) and mouse (EST, AA139022) counterparts of the rat AROM gene. BAC clones bearing sequences of the human AROM gene were also spotted. Individual cDNA or genomic clones were queried and sequenced. Exon-intron junctions of the AROM gene were sequenced and found at similar positions in rat, mouse, and human genomic DNAs. By combining all these sequence data, a map of the exon-intron organization of the AROM gene can be proposed. The rat AROM gene illustrated in Fig. 2A spans about 34 kb, contains at least 12 exons, and encodes a large spectrum of RNA molecules, some with putative protein-coding portions and others likely encoding transcripts (Fig. 2B). Two putative cap sites based on RACE experiments were located in the 5′ end of the AROM gene upstream of the largest open reading frame, and they were designated CS1 and CS2. Transcription of short uncoding RNAs may be initiated at the potential internal cap sites named CS3, CS4, and CS5, respectively. Three polyadenylation sites were mapped in exon 12 of the AROM gene, i.e. in the opposite part of intron A and of the regulatory sequences of the MCH gene.

Alternative splicing of exons of the AROM gene produces at least four distinct RNA species that may potentially encode proteins. An exon 3-spliced transcript of about 4.0 kb in length encodes a putative protein of 573 amino acids (aa) designated AROM-p64 which corresponds to the largest protein that could be generated from the AROM gene according to our present data (Figs. 2B and 3). The ATG initiation codon of AROM-p64 fits perfectly with the consensus motif (24). Further RT-PCR analyses indicated that polyadenylation site 3 of the AROM gene was associated with the AROM-p64 transcript. Skipping of exons 3 and 11 results in a truncated version of AROM-p64, named AROM-p50, with a different C terminus due to an open reading frameshift (Figs. 2B and 3). Insertion of exon 3 in one AROM gene transcript introduces a stop codon shortly after the initiation codon of AROM-p64 and leads to the production of a potential 7-kDa protein (Fig. 2B). However, several translational initiation codons, with favorable Kozak’s sequence context, are found in the same reading frame as AROM-p64. Therefore, a putative protein named AROM-p54 may initiate at a downstream ATG codon then the one used to produce the AROM-p64 and appears identical to AROM-p64 for the remaining part (Fig. 2B). Finally, transcripts that initiate at the putative CS2 would allow the synthesis of a protein named AROM-p17, identical to the C terminus of AROM-p64 (Fig. 2B). Sequence analysis of rare RACE products revealed also the sporadic deletion of codon CAG at position 1357 in some AROM-p64 mRNA due to alternative splicing (inverted open arrowhead, Fig. 3). It was not possible to assign precise polyadenylation sites to AROM-p50 mRNA, AROM-p54 mRNA, and AROM-p17 mRNA.

In addition to the coding AROM mRNAs, we found a class of three putative antisense RNAs overlapping mainly the coding portion of the MCH gene and starting at cap sites CS3, CS4, and CS5, respectively (Fig. 2B). It is worth noting that the overt expression of these unspliced and uncoding transcripts remains at this stage hypothetical. Indeed, we cannot exclude artificial pausing of the reverse transcriptases during synthesis of the large cDNA products that share with the short unspliced RNA the same sequences in their 3′ end parts. It was also not possible to assign a precise polyadenylation site to these individual RNAs.

The AROM p64 and Related Proteins Displayed Sequence Similarities with DNA/RNA-binding Proteins—Sequence analyses, using the PSORTII computer program, indicate that AROM-p64 lacks a peptide signal and/or transmembrane domain but is likely to be a nuclear protein (prediction at 52.2% in the k-NN test). Consistent with this hypothesis, a leucine zipper-like motif was found conserved among mouse, rat, and human AROM-p64 at residues 240–272 (Fig. 4, A and C). This
segment of the AROM-p64 could exist in an α-helical conformation. According to the leucine-zipper model (25), one side of the hypothetical helix is composed of hydrophobic residues (three leucines and one isoleucine), and the other appears rich in amino acids with charged and uncharged polar side chains.

Another intriguing aspect in the N terminus sequence of AROM-p64 was the presence of an RNA recognition motif.
(RRM). This RRM consists of a β1-α1-β2-β3-α2-β4 structure with two submotifs designated RNP-1 and RNP-2, highly conserved among RNA-binding proteins (26, 27) (Fig. 4B). The sequence between aa 188–290 of rat or human AROM-p64 fitted quite well with the consensus RRM structural core sequence. In particular, the most obvious signature for canonical RRM, i.e., the RNP-1 submotif, was fairly well conserved in AROM-p64 (Fig. 4B). However, sequence alignments displayed major differences with other RRM in the RRM-like domain together with an N-terminal RRM suggest that AROM-p64 with a domain of bacterial helicases (Fig. 4C). The positions of conserved residues are highlighted by gray shading. Black shading corresponds to strongly conserved residues in SR proteins. The RNP-1 and RNP-2 submotifs are indicated. Brackets for loop 2 and loop 4 underlined the absence of consensus using a Chou and Fasman’s secondary structure model. C, partial alignment of human (h) AROM-p64, mouse (m) AROM-p64 and rat (r) AROM-p64 sequences and selected bacterial helicases. Full identities are indicated in dark shading. Gray shading represents conservative substitutions. The consensus leucine-like motif is indicated by asterisks on AROM-p64, and it is shown below the alignment. –, gaps.

Interestingly, several charged (lysine or arginine) and serine residues were located at the C terminus. In particular 9 SK/SR doublets were found between residues 390 and 510 (large letters in bold, Fig. 3). The presence of a C-terminal RS-like domain together with an N-terminal RRMM suggest that AROM-p64 and related proteins may be new members of the splicing factor family (28).

Finally, we revealed 30% sequence identities (58% sequence similarities) between a central portion of the mammalian AROM-p64 with a domain of bacterial helicases (Fig. 4C). Highly conserved motifs “U(S/A)Y(L)XLLUXUX24N(D/D/L)AALU” and “(U/V)LV(T/D)PRKG(L/D)G” (U = uncharged residues) were found in AROM-p64 and helicases. However, comparison of the AROM-p64 sequence with the bacterial or mammalian helicase family did not reveal other regions of homology. In particular, AROM-p64 failed to possess the seven amino acid motifs that are shared by all DNA/RNA helicases (reviewed in Refs. 29 and 30).

Tissue-specific Expression of the Rat AROM Gene—Distribution of AROM mRNAs in adult rat organs was examined first by RT-PCR, using oligo(dT) for reverse transcription and primer 26 or 24 as a forward primer and primer 16 as a reward primer (Fig. 5, A and B and Table I). The highest levels of AROM gene transcripts were found in testis and then in the gastrointestinal tract (jejunum, ileum, and colon) and immune tissues (spleen and thymus). Weak expression was also found in the lung, kidney, pituitary gland, and muscle. In sharp contrast, AROM mRNAs were apparently missing in the rat hypothalamus, where the MCH gene is actively expressed (31), and in peripheral tissues such as the heart and liver. By using the specific primer RMCH 9 to perform reverse transcription and 16/26 primers for the PCR (Fig. 5A), we observed a pattern identical to this shown in Fig. 5B (bottom panel), demonstrating that we characterized overt antisense MCH RNAs (not shown). Sequence analysis revealed that the major RT-PCR product of 1300 bp found with the 16/26 primers corresponded to AROM-p64 mRNA.3

The patterns of expression of the AROM mRNAs was compared next in the rat testis and PC12 cells using primer 24 as a forward primer and a set of primers selected at different locations in exon 12 of the AROM gene (see Fig. 5A). As shown in Fig. 5C, the expression of AROM gene transcripts appeared quite similar in PC12 cells and rat testis. However, when primers located downstream from polyadenylation sites 1 and 2 were used, the pattern was clearly distinct, and the lengths of RT-PCR products indicate multiple processing events between exon 11 and the 3‘ end of exon 12 of the AROM gene (right panel, Fig. 5C). This suggests a differential regulation of poly(A) 3-site-containing AROM mRNA synthesis/splicing in the PC12 cells and rat testis.

Expression of AROM Proteins in Baculovirus-infected Sf9 Cells, Adult Rat Testis, and PC12 or HeLa Cells—To obtain antibodies that allowed selective recognition of the N- and C-terminal parts of AROM-p64 and related proteins, PCR products encoding predicted rat AROM-p50 and AROM-p17 (see Fig. 2B) were individually subcloned into the E. coli vector pGEX-3, and the fusion proteins were injected in rabbits (32). The resulting polyclonal antisera were named Ol 72 for Ab specific to AROM-p50 (sharing the same N terminus with AROM-p64) and Ha 43 for Ab detecting AROM-p50 (Fig. 5D).

3 F. Presse, L. Borsu, and J.-L. Nahon, manuscript in preparation.
PCR products encoding the predicted mouse AROM-p64 and rat AROM-p50 were subcloned into the baculovirus expression vector pVL, and Sf9 cells were infected with the recombinant virus. Protein extracts from the infected Sf9 cells were analyzed by Western blotting using Ol 72 Ab or Ha 43 Ab (Fig. 6A).

Ol 72 Ab recognized a major band of 50 kDa and a weak band of 64 kDa (black arrowheads) in Sf9 cells expressing, respectively, AROM-p50 (lane p-50) and AROM-p64 (lane p64). Two minor bands were also identified in the lane p64 (white arrowheads in Fig. 6, A–D) and could either represent degradation products of AROM-p64, translational products of the AROM-p64 initiated at downstream initiation sites, or unrelated proteins carrying epitopes recognized by Ol 72 Ab. Ha 43 Ab detected also the 64-kDa protein in infected Sf9 cells. As expected, AROM-p50 was not revealed with Ha 43 Ab (left panel, lane p50), but multiple proteins were detected in both protein extracts (lanes p64 and p50), indicating that epitopes recognized by Ha 43 Ab are shared by proteins expressed by infected Sf9 cells.

Western blot analysis was performed with whole cell extracts of unstimulated PC12 cells or adult rat testis (Fig. 6B). Ol 72 Ab identified the same 64-kDa protein in AROM-p64 expressing Sf9 cells (lane p64), rat testis (lane testis), and PC12 cells (lane PC12). Minor bands were revealed in AROM-p64 ex-
pressing Sf9 cells and PC12 cells (white arrowheads, Fig. 6B).

Ha 43 Ab revealed the 64-kDa protein in PC12 cells and, at a lowest level, in the rat testis. Additional bands in the range of 50–120 kDa were found with this antiserum in the rat testis, some of them being present in Sf9 cellular extract (lane p64).

Intracellular analysis of AROM protein expression in PC12 cells is illustrated in Fig. 6C and Fig. 7. The Ol 72 Ab detected the 64-kDa protein in majority in the cytoplasmic extract (lane PC12C). This protein was also found weakly expressed in a nuclear extract (lane PC12N). The two minor bands found in AROM-p64-expressed Sf9 cells were found in the nucleus (lane PC12N). The same proteins were barely visible with the Ha 43 Ab in a nuclear extract of PC12 cells (lane PC12N, left panel). In agreement with the Western blot data, predominant cytoplasmic staining was found by immunohistochemistry analysis of PC12 cells with Ha 43 Ab (Fig. 7A) and Ol 72 Ab (Fig. 7C). As controls, background signal was revealed with the preimmune serum (Fig. 7B) and strong expression of the tyrosine hydroxylase was confined to the cytoplasm (Fig. 8D).

S100 nuclear extracts from HeLa cells provide a widely used system for studying RNA-binding proteins and particularly SR-splicing proteins. To characterize further AROM proteins in mammalian cells, we performed immunoblot of a HeLa cell nuclear extract. The Ol 72 Ab identified two bands of 64 (black arrowhead) and 55 kDa (white arrowhead) in AROM-p64-expressing Sf9 cells (lane p64) and in a nuclear extract of HeLa cells (lane HeLaN, Fig. 7D). The same proteins as well as many others were recognized by Ha 43 Ab. This demonstrate that AROM proteins could be identified in the nucleus of HeLa cells.

**Time Course of AROM and MCH Gene Expression in PC12 Cells**—Northern blot analysis with total RNAs of PC12 cells grown for 0.5 to 24 h in the presence of NGF and lithium revealed a reciprocal regulation of the 4.0-, 3.5-, and 1.4-kb antisense MCH gene transcripts and MCH mRNA during the first 6 h of treatment (Fig. 8A). The 4.0/3.5-kb mRNAs were transiently up-regulated at 1 h, became undetectable at 6 h (and 12 h; Fig. 1), and reached about the same levels as in untreated cells at 24 h. The content of the 1.4-kb mRNA decreased slightly during the first 6 h of NGF/lithium treatment and was similar to that of control cells at 12 and 24 h. Conversely, mature MCH mRNA was absent up to 3 h, clearly visible at 6 h, and strongly expressed at 12 and 24 h. A difference in the length of the poly(A) tail could account for the higher size of MCH mRNA found at 6 h.4 As a control, GAPDH mRNA level remains nearly the same during the time course treatment.

The expression of MCH and AROM mRNA was further ex-

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Fig. 6. Western blot analysis of AROM proteins. A, antisera raised against AROM-p50 (Ol 72 Ab; 1:1000 final) and AROM-p17 (Ha 43 Ab; 1:500 final) were used to probe Western blots containing 4 μg of protein extract from AROM-p64 (lane p64) or AROM-p50 (lane p50) expressing Sf9 cells. B, 10 μg of AROM-p64 expressed in Sf9 cells and 40 μg of proteins extracted from adult rat testis (lane testis) or PC12 cells (PC12T) were analyzed by Western blotting. C, subcellular distribution of AROM proteins in PC12 cells. Immunoblotting with Ol 72 Ab or Ha 43 Ab was performed as in A with 40 μg of protein extracts. PC12N, nuclear proteins; PC12C, cytoplasmic proteins. D, nuclear localization of AROM proteins in HeLa cells. 10 μg of AROM-p64 extracted from Sf9 cells and 50 μg of nuclear extract of HeLa cells were loaded, and Western blot analysis was carried out like in A. The black arrowheads indicate the 64- and 50-kDa protein. The white arrowheads correspond to proteins found with Ol 72 Ab in cellular models and tissues expressing AROM-p64 protein.

Fig. 7. Subcellular localization of AROM proteins. Unstimulated PC12 cells were grown on coverslips, and immunostaining was performed with Ha43 Ab (A), a preimmune serum (Pre-IS, B), Ol72 Ab (C), and an anti-tyrosine hydroxylase Ab (TH Ab, D). Note the staining of cytoplasms in A, C, and D. Magnification, × 20.

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4 L. Borsu, F. Presse, and J.-L. Nahon, unpublished data.
Binding specificity was assessed by performing the binding reaction in the presence of calf thymus DNA competitor. Under this condition AROM-p64 was not detected at any KCl elution steps nor in the final protein extraction from beads, indicating that this protein is not a DNA-binding protein, we performed binding assays with denatured calf thymus ssDNA coupled to agarose or cellulose resin. Mouse AROM-p64 was produced in SF9 cells using baculovirus infection. Crude pellet extracts were incubated at low ionic strength in two different buffers (A and B; "Experimental Procedures") with ssDNA-coupled resin; bound proteins were eluted with increasing KCl concentrations (0.2 to 1 M) to assess the binding strength, and AROM-p64 was revealed by SDS-PAGE using OI 72 antiserum. About 65% of AROM-p64 binds strongly to ssDNA-agarose resin (~55 kDa), and AROM-p64 was revealed by SDS-PAGE using either of binding conditions (buffer A or B, middle panel). Unfortunately, AROM-p17 could not be synthesized in the SF9 cellular model. As a negative control, a secreted protein named MGOP (32) was produced in the SF9 cells.

Fig. 8. Time course of MCH and AROM mRNA expression during NGF and lithium treatment. A, Northern blot analysis. Two RNA samples (A and B, 20 μg/lane) from various times after NGF/lithium addition to PC12 cells were separated electrophoretically, bound to Hybond N filter, and hybridized successively with MCH and GAPDH 32P-labeled cDNA probes. Mature MCH mRNA and AROM gene transcripts are indicated as in Fig. 1. B, RT-PCR analysis. Total RNA samples (n = 4; two are shown in A and B) were extracted from PC12 cells treated with NGF/lithium, reverse-transcribed with oligo(dT), and amplified with the primers noted on the right. The amplified products were revealed with an MCH cDNA probe (upper panel) or an AROM-specific cDNA probe (middle and bottom panels). The lengths of the RT-PCR products are indicated on the right. The non-reverse-transcribed samples were all negative (not shown). C, Western blot analysis. Protein extracts (n = 4; two are shown in A and B) were isolated from PC12 cells treated with NGF/lithium for the indicated times and loaded (40 μg/lane) on a 10% SDS-PAGE gel. Immunoblot was carried out with OI 72 Ab and then probed again with MAPK Ab (R23 Ab, Santa Cruz Biotechnology). Positions of AROM proteins are indicated as in Fig. 6.
extracted, and tested under the same conditions as AROM proteins. This protein did not bind to ssDNA-agarose (Fig. 9B, bottom panel). These results supported the view that AROM-p64 selectively recognized single-stranded DNA in vitro.

To characterize further the nucleic acid-binding properties of AROM-p64, we tested the bindings of recombinant proteins produced by Sf9 cells to ribonucleotide homopolymers conjugated to agarose or Sepharose beads. As shown in Fig. 10A, AROM-p64 binds to RNA homopolymers with various affinities showing a relative selectivity for poly(A). The resistance of AROM-p64-poly(A) interaction up to 0.6–0.8 M KCl concentration (Fig. 10, A and B) and association of AROM-p64 to poly(A), poly(G), and to a lesser extent to poly(C) beads suggested that hydrophobic contacts could be involved in this complex formation. Furthermore, heparin at 1 mg/ml was a full competitor for binding of AROM-p64 to poly(A), whereas tRNA was not. This suggests that retention of AROM-p64 to poly(A) results also to interactions to the polyphosphate backbone.

**DISCUSSION**

We have identified a new gene named AROM that appears to be encoded by the opposite strand at the same locus as the MCH gene. In PC12 cells, two classes of AROM gene transcripts can be distinguished on the basis of the RACE-PCR experiments as follows: 1) potential unspliced RNAs complementary to the exonic-intronic regions of the MCH gene, but RNase protection analyses are required to confirm the locations of the corresponding putative cap sites; 2) multiple spliced variant RNAs, mainly complementary to the 3’-flanking part of the MCH gene and encoding new DNA/RNA-binding proteins. Antisense transcription of the MCH gene was originally reported in rat gut (33) and human hypothalamus (34). However, in both cases only spliced RNAs complementary to the coding regions of the MCH gene were reported. This suggests that additional antisense MCH RNAs may be produced in vivo and remain to be characterized.

The results showing that the large transcripts hybridizing to MCH cDNA probe correspond to antisense MCH RNAs supported our previous data (16) suggesting a post-transcriptional regulation of the MCH gene following a NGF/lithium treatment of PC12 cells. Indeed, an unexpected rise in MCH mRNA content was observed when a transcriptional inhibitor was applied to PC12 cells treated with NGF/lithium for 24 h, i.e. at a time when MCH mRNA begun to decrease and AROM 4.0/3.5-kb RNAs reciprocally increased (16, 17). Functional relationship between the MCH and the AROM genes is strengthened by the time course studies of the NGF/lithium treatment in PC12 cells which demonstrated a reciprocal relationship between MCH/AROM mRNA (and protein) expressions at the time of MCH mRNA induction (Fig. 8). Interestingly, the largest AROM gene...
transcripts were totally absent at the time of induction and maximum expression of the MCH mRNA, i.e. 3 and 12 h, respectively, after NGF/lithium stimulation of PC12 cells (Figs. 1B and 8B). Therefore, it is tempting to speculate that transcriptional shut down of antisense MCH RNA synthesis may lead to an increase in MCH mRNA stability. Alternatively, the ratio of sense to antisense RNA may be the “sensing indicator,” and transcriptional activation of the MCH gene upon NGF/lithium stimulation would overcome the inhibitory effects of antisense RNAs. Formation of sense/antisense RNA complexes may induce base modifications and subsequent degradation as illustrated in the case of the basic fibroblast growth factor (bFGF) transcript during maturation of the Xenopus oocytes (35) or as recently suggested for the human PCNA gene (36).

However, we do not have any evidence of the interaction of the sense and antisense MCH RNAs. Alternatively, the translation products of the AROM gene may be involved in the regulations of production and/or stability of the MCH mRNA.

The most striking aspect of our work is the demonstration that some of the AROM RNAs encoded a family of new proteins generated by alternative splicing. It is worth noting that the coding part of the AROM gene identified so far does not overlap with the coding part of the MCH gene (see Fig. 3A). However, both genes are physically linked on human chromosome 12q23,2 indicating that the protein-encoding AROM gene mRNAs are not generated by a trans-splicing mechanism. Based on the RACE-PCR and RT-PCR experiments, at least four transcripts that may encode AROM-p64, AROM-p54, AROM-p50 and AROM-p17, respectively, were found in PC12 cells and in the rat tissues (see Figs. 2 and 5B). The translational capabilities of AROM-p64 and AROM-p50 mRNAs were further confirmed by translation experiments using recombinant baculovirus-infected Sf9 cells and in cell-free systems.4

The AROM proteins convey, or not according to the extent of overlapping with AROM-p64, two distinct putative domains as follows: a putative RNA/DNA binding domain located at the N terminus and an SR/SK-rich domain located at the C terminus. The SR/SK-rich domain was rather suggestive that AROM-p64 and derived AROM proteins sharing this domain could belong to the superfamily of splicing or spliceosome-associated factors (28, 37). AROM-p64 and other putative AROM proteins were found in HeLa cell nuclear extracts (see Fig. 6D). Preliminary experiments using these extracts immunodepleted with OI 72 Ab or Ha 43 Ab did not reveal any effects on in vitro splicing of β-globin RNA precursor.5 This suggests that AROM proteins found in the nucleus of HeLa cells are not essential for constitutive splicing. However, this does not preclude a role for these proteins in splicing of restricted pre-mRNA substrates. Interestingly, SR motifs were also found in the C-terminal domain of AROM-p50 but embedded within a distinct C terminus (see Fig. 3). Divergence of the SR/SK domain between AROM-p64 and AROM-p50 might modify the protein-protein interaction properties with other factors containing the same motifs as documented previously (37). It is worth noting that the number of the canonical SR pairs of amino acids remains low in AROM-p64 or AROM-p50 (3 out of 9 serine/basic residues repeats) and are rather scattered along the C-terminal part. Accordingly, none of these proteins produced in baculovirus systems were detected by monoclonal antibody 104 on Western blots (data not shown). This antisera cross-reacts with the phosphorylated forms of the SR proteins (38), excepted the ones that carry few SR repeats (39).

Another notable feature of SR proteins is the N-terminal RNP-type RRM (26). A region reminiscent of that domain was identified between residues 188 and 290 of AROM-p64 and spliced product homologues (Fig. 4B). The residues critical for structure (in general buried hydrophobic amino acids) were highly conserved in RNP-1 and RNP-2 motifs. Strong conservation was also noted at the RRM Phe56 residues in RNP-1 (counterpart of the Phe254 of AROM-p64) which appears essential for RNA binding (26). On the contrary, other exposed residues important for the functional specificity of the SR proteins were rather divergent (for instance, aromatic residues present in RNP-2). These and other differences in the lengths of the α-helices and β-turns of the AROM proteins might be crucial for the RNA binding or protein-protein interaction selectivity.

In addition to structural relationship with RNA-binding proteins, a conserved pattern identified in bacterial DNA/RNA helicases and a putative leucine-zipper motif were found adjacent to the central region of AROM-p64 (see Fig. 4, A and C). The region of sequence identity with the helicases falls in a space of variable length between motifs IV and V of bacterial superfamily II helicases (reviewed in Ref. 40). The functional importance of this helicase domain is not currently known. The effect of point amino acid substitutions in the sequence 403DDAAFER409 of the bacterial helicase UvrD (see Fig. 4C) leads to modification in the unwinding activity (41). However, AROM-p64 and derived proteins lack the seven conserved motifs shared by all helicases and particularly the A and B motifs of the Walker-type NTP-binding pattern required for NTPase activity (40). Therefore, it is unlikely that the AROM proteins function as genuine helicases. Conceivably, structural similarity may be related to the RNA/DNA-binding properties of helicases. Therefore, AROM-p64 contains some of the typical features of DNA/RNA binding factors. In agreement with this hypothesis, we showed that AROM-p64 produced in infected Sf9 cells may interact with ssDNA and RNA homopolymers with some selectivity for poly(A). Ionic contacts at the base level (dissociation from ssDNA and poly(A) columns at 0.6–0.8 M KCl) as well as hydrophobic interactions (resistance of protein/RNA interactions at high ionic strength) and electrostatic interactions (sensitivity to heparin competitor) with the polyphosphate backbone participate with the AROM-p64/ssDNA/RNA binding. However, we cannot completely rule out that AROM-p64 is retained on the ssDNA and RNA column indirectly because of strong interaction with a true nucleic acid-binding protein present in the infected Sf9 cells. This should be solved by using NorthWestern analysis with purified AROM-p64. Furthermore, recombinant AROM-p50 did not bind to either ssDNA (Fig. 9C) or RNA homopolymer columns.2 In addition, co-incubation of AROM-p64 and AROM-p50 did not modify the binding of AROM-p64 to poly(A) column (not shown). This indicates that the putative RM/DNA binding domain shared by AROM-p64 and AROM-p50 is not essential for binding activity and that the highly charged C-terminal part of AROM-p64 could be responsible for the protein-nucleic acid interactions. This needs to be addressed now using AROM-p64 deletion constructs.

Western blot analysis and immunohistochemistry demonstrated that AROM-p64 and other AROM proteins are mainly localized into the cytoplasm of unstimulated PC12 cells. However, these proteins could also be found in the nucleus of PC12 cells and HeLa cells. Although canonical nuclear localization or export motifs were not identified in AROM-p64, a very basic sequence in the C-terminal part (aa 494–525) and a hydrophobic leucine-rich sequence in the N-terminal part (aa 70–80) could function as nuclear localization and nuclear export signal, respectively. Interestingly, a shift between two isoforms of AROM proteins, AROM-p64 and a putative nuclear-specific

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5 L. Borsu and J. Marie, unpublished data.
AROM protein, was found in PC12 cells following NGF/lithium treatment (see Fig. 8C). It is tempting to speculate that a balance between AROM protein variants, differentially distributed in the nucleus or cytoplasm, could direct changes in RNA metabolism, transport, or stability associated with differentiation of PC12 cells.

There are now a number of examples of bidirectionally transcribed eukaryotic genes for which a function has been proposed (42). Different classes of antisense RNAs can be defined. 1) Antisense RNAs that regulate the level of the complementary RNA in forming hybrid and inducing RNA editing and degradation processes. The most studied models are the antisense bFGF gene in Xenopus (34) and the rat bFGF antisense gene which encoded also a novel protein with sequence identity to the MuT gene products (43). 2) Non-coding antisense RNAs that may inhibit splicing of the complementary DNA by base pairing, as illustrated by the rat erbB locus (44) and as proposed for the short antisense RNA overlapping the exon 1-intron 1 junction of the human PCNA gene (36). 3) Antisense RNAs may compose coding sequences that apparently are unrelated to the proteins encoded by the sense gene transcripts. Indeed, a pupal cuticle protein is located within the intron of the Gart gene, encoding purine pathway enzymes in Drosophila melanogaster (45). 4) Antisense RNAs may encode proteins, with structural or functional relationships with the polypeptide coded by the sense transcripts. For instance, the N-cym gene can produce a basic helix-loop-helix transcriptional factor like the complementary N-myc gene (46). Another example is the PR264/SC35 splicing factor that could participate in the splicing of its complementary exon of thymic RNA sequence and the c-myb mRNA in avian thymic cells (47).

To our knowledge, the simultaneous expression of non-coding unsplliced RNA complementary to a coding gene and coding spliced variant RNAs partially overlapping the sense gene has not been described previously. In addition the MCH/AROM gene system represents a rather rare example of overt reciprocal expression in a cellular model (see Fig. 8) and also in developing tissues.3 Formation of a double-stranded structure by the complementary MCH RNAs could inhibit the MCH mRNA production at the level of RNA processing and/or stability. Furthermore, we proposed that AROM proteins could control gene transcript production (including MCH mRNA) either at the level of transcription if AROM-p64 and derivatives are DNA-binding proteins or at the level of post-transcriptional processing, transport, and stability if these proteins are mRNA-binding factors as suggested by our presented data.

Recently, MCH-deficient (MCH−/−) mice were produced by homologous recombination and exhibited striking reduction of body weight due to hypophagia and increased metabolic rate (11). The phenotypes of MCH−/− mice were attributed to the absence of the orexigenic MCH peptide. However, these mice carried a PGK-neo cassette instead of the region encompassing the exons I–III of the MCH gene. It is now of obvious interest to determine whether the presence of the PGK-neo and/or deletion in the putative 3’ end-untranslated region of the AROM mRNAs results in any changes of AROM gene expression associated with modifications of feeding behavior (and other functions) in the MCH−/− mice.

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