Production of rat soluble and membrane-bound catechol *O*-methyltransferase forms from bifunctional mRNAs

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In the rat, the catechol *O*-methyltransferase (COMT) gene has been found to contain two promoters, P1 and P2. This organization enables the gene to produce a soluble (S-COMT) and a membrane-associated (MB-COMT) protein by using two in-frame ATG initiation codons (S- and MB-ATG). The P1 promoter expresses a 1.6 kb transcript (S-mRNA) which codes for the S-COMT polypeptide only. Here we demonstrate that the P2 promoter controls the expression of alternatively spliced 1.9 kb transcripts (MB-mRNA) which differ by a 27-nucleotide region immediately upstream of the MB-AUG codon. The presence of the 27-base sequence alters the nucleotide at position −3 from G to C, thereby changing the translation initiation context of the MB-AUG codon. Expression experiments in COS-7 cells using full-length COMT cDNAs showed that this alteration affected the initiation of the translation of the MB-AUG and consequently changed the relative amounts of MB- and S-COMT polypeptides produced. No proteolytic cleavage of the MB-COMT form to S-COMT was detected in *in vitro* or *in vivo* pulse–chase experiments. We conclude that the bifunctional 1.9 kb mRNAs are able to produce both S-COMT and MB-COMT polypeptide by the leaky scanning mechanism of translation initiation.

INTRODUCTION

The enzyme catechol *O*-methyltransferase (COMT; EC 2.1.1.6) inactivates substrates containing a catechol structure by methylating one of the phenolic hydroxy groups in mammals [1]. These substrates include catechol hormones and neurotransmitters, such as dopamine and adrenaline [2].

All mammalian tissues studied contain two forms of the COMT protein. Most of the activity is due to the cytoplasmic form of the enzyme (S-COMT), whereas the membrane-bound form (MB-COMT) is responsible for a smaller proportion [3–6]. Both protein forms have been characterized from rat and man. The molecular mass of S-COMT is 24 kDa in rat [7] and 25 kDa in man [8], and that of the larger form, MB-COMT, is 28 kDa [6] and 30 kDa [6,9] in rat and man respectively. Although S- and MB-COMT have similar biochemical properties, MB-COMT differs structurally from S-COMT by having a hydrophobic extension (43 amino acids in rat and 50 amino acids in man) at the N-terminus. This region contains a signal-anchor peptide directing and anchoring the MB-COMT polypeptide to cell membranes [10].

COMT cDNAs have been cloned from rat liver [11], porcine liver [9], human placenta [12] and one human cell line [9]. Results from these studies strongly suggest the presence of only one gene for COMT in rat and man. The structure of the rat COMT gene has been characterized [13]. It contains an S-ATG codon in the second exon and an MB-ATG codon 129 bp upstream for the translation initiation of S-COMT and MB-COMT polypeptides respectively. The coding sequence for 221 amino acids downstream of the S-ATG codon is identical for both enzyme forms, and the region between the ATG initiation codons encodes the signal-anchor peptide. This organization enables the production of two COMT forms from one gene. The gene is regulated by two promoters, P1 and P2, P1 expressing a 1.6 kb transcript (S-mRNA) containing only the S-AUG translation initiation codon of S-COMT. The P2 promoter expresses 1.9 kb transcripts (MB-mRNA) containing both AUG initiation codons and having a coding capacity for both enzyme forms.

According to our previous work [13] and the present report, only the 1.9 kb MB-mRNA species can be detected in rat brain. However, both COMT proteins can be found in the brain [3,4,6]. We have proposed that the leaky scanning mechanism of translation initiation [14,15] is responsible for the production of S- and MB-COMT forms from this single mRNA species in the rat brain and other tissues [10,13]. Also, the involvement of proteolytic processing has been suggested [16].

We report here that, in rat, the bifunctional 1.9 kb MB-mRNA is able to produce both S- and MB-COMT forms by using alternative AUG initiation codons. Analyses of 5′-end-specific cDNA clones show that the 1.9 kb MB-mRNA class consists of alternatively spliced species in which the effectiveness of the MB-AUG codon in initiating translation is different. We also provide evidence suggesting that, under the experimental conditions used, no proteolytic cleavage of the MB-COMT to the S-COMT form occurs.

MATERIALS AND METHODS

Western-blot analysis

Selected rat tissues were disrupted in PBS, and 25–50 μg of total proteins subjected to SDS/PAGE (10% gels). Western blotting was performed as described previously [7] using polyclonal antiserum raised against the recombinant S-COMT polypeptide.

Northern-blot analysis

Total RNA was extracted from different rat tissues and analysed by Northern blotting as described previously [13]. The probe

Abbreviations used: COMT, catechol *O*-methyltransferase; S-COMT, MB-COMT, soluble and membrane-bound forms of COMT.

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All nucleotide sequences described here will appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under the accession numbers Z12651 and Z12652.

Table 1 Oligonucleotides used in the present study

All nucleotide positions refer to the published rat COMT gene sequences [13].

Name	5′-3′ COMT-specific sequence	Location
PR1	CAGCTAGGAGCACTGTC	+618–+634
PR2	CAGATGCCGTTGGCTGCAGTCTC	−3–+20
PR3	AGGTGTCGCAAGAGCAGGAGGAGGGC	+40–+65
PR4	CAACAACAGGAGAC	+26–+39
PR5	GTGTTAAAACCCGTGTCTG	+52–+70
PR6	TTTCGCGTCACCCACATTCATGG	+245–+267
PR7	AAGATGGCGTTGGCTGCAGTCTCATT	−3–+23

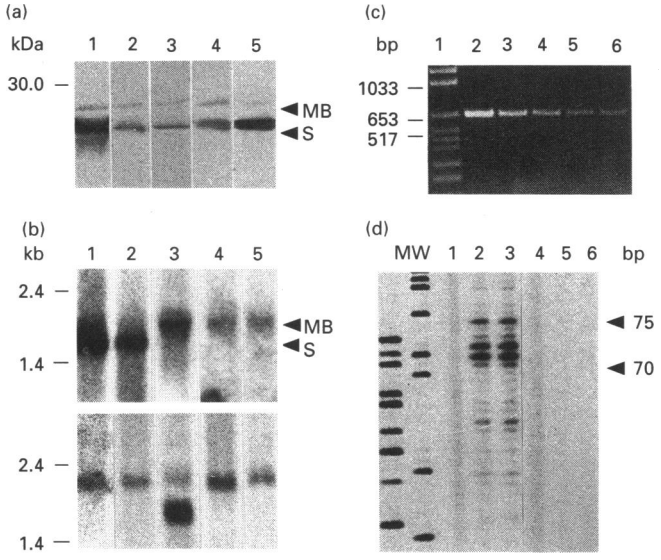

Figure 1 Characterization of COMT proteins and transcripts in rat tissues

(**a**) Western-blot analyses of rat tissues. After disruption of the tissues in PBS, proteins were subjected to SDS/PAGE (10% gels), and Western blotting was carried out as described [7] using polyclonal anti-COMT serum. COMT polypeptides were detected by using a second antibody conjugated with horseradish peroxidase. Lane 1, liver; lane 2, kidney; lane 3, heart; lane 4, telencephalon; lane 5, cerebellum. The positions of a molecular-mass standard and 24 kDa S- and 28 kDa MB-COMT polypeptides are indicated. The amounts of total proteins used were 25 μg for the liver and 50 μg for the other tissues. (**b**) Northern-blot analysis of rat COMT mRNA. Total RNAs were purified, electrophoresed in a 1.2% formaldehyde/agarose gel, and blotted on to a Hybond-N filter as described previously [13]. The filter was hybridized with a ^{32}P-labelled fragment (a 209 bp *Eco*RI–*Pst*I fragment) of the rat S-COMT cDNA clone (upper panel) or with a ^{32}P-labelled rat β-actin probe (lower panel). Lane 1, liver; lane 2, kidney; lane 3, heart; lane 4, telencephalon; lane 5, cerebellum. The positions of molecular-mass standards (on the left) and 1.6 kb S- and 1.9 kb MB-mRNA (on the right) are indicated. The amounts of RNAs used were 2.5 μg for the liver and 10 μg for the other tissues. (**c**) PCR analyses of rat total RNAs. After cDNA synthesis, a conventional PCR was carried out using primers located in exon 5 (PR1) and overlapping MB-ATG codon (PR2). Then 10% of the products were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide (0.5 μg/ml). Lane 1, molecular-mass markers; lane 2, liver; lane 3, kidney; lane 4, heart; lane 5, telencephalon; lane 6, cerebellum. (**d**) Nuclease-protection assays of the rat liver and telencephalon poly(A)$^+$ RNAs. The assays were carried out as described previously [13] using probes NP2 and NP3 to detect the putative transcription initiation and/or splicing sites near the COMT S-AUG codon. Lanes MW, sequencing ladders; lane 1, heterologous RNA (yeast tRNA) + NP2; lanes 2 and 3, liver poly(A)$^+$ RNA + NP2 and NP3 respectively; lane 4, heterologous RNA (yeast tRNA) + NP3; lanes 5 and 6, telencephalon poly(A)$^+$ RNA + NP2 and NP3 respectively. The positions of the protected fragments between 70 and 75 bp are indicated on the right.

used was the ^{32}P-labelled 5′ fragment (a 209 bp *Eco*RI–*Pst*I fragment) of rat S-COMT cDNA [11]. Rat β-actin transcripts were analysed to estimate the quantity and integrity of RNAs.

PCR analyses of total RNAs

The first-strand cDNAs were synthesized from 150 ng of total RNAs with the Riboclone cDNA Synthesis System (Promega Corp., Madison, WI, U.S.A.). The downstream primer in the cDNA synthesis and the following PCR reactions was PR1 (Table 1 and Figure 3a below), located in exon 5 of the COMT gene. The cDNAs were amplified by conventional PCR procedures with Taq DNA polymerase (Promega) using PR2 as the upstream primer.

Nuclease protection

To reveal the 5′ ends of COMT transcripts near the S-AUG codon in the rat liver and telencephalon poly(A)$^+$ RNAs, RNAase-protection assays were carried out. The RNA probes used were NP2- and NP3-containing nucleotides from −630 to +183 and from −413 to +183 respectively of the rat genomic COMT sequence [13].

Amplification of the 5′ end of the COMT mRNA

The amplification of the 5′ end of the rat heart COMT cDNA has been described previously [13]. The COMT-specific primers PR3 and PR4 downstream of the MB-ATG codon were used in the cDNA synthesis and PCR reactions respectively (see Table 1 and Figure 3a). The clones derived from the tailing reactions were screened with a 39 bp ^{32}P-labelled COMT-specific fragment (positions +1 to +39; Figure 3a). A total of 40 COMT-specific clones were characterized by sequencing.

Construction of the full-length cDNA clones

The 5′-end-specific cDNA fragments derived from the tailing reactions were amplified as follows. The fragments without or with a 27 bp region which is located upstream of the MB-ATG codon were used as templates. The downstream primer was PR4 (Figure 3 and Table 1) in exon 2 of the COMT gene. The upstream primer, containing a *Sac*I site in the 5′ end, was PR5 in the non-coding exon 1. The resulting PCR fragments were then used as megaprimers [17] to amplify a fragment from a rat genomic DNA clone. In these cases, the downstream primer was PR6 within the S-COMT coding region. This primer contains an internal *Bst*XI site. The *Sac*I–*Bst*XI fragments of the PCR products were joined to the *Bst*XI–*Hin*dIII fragment of the rat liver COMT cDNA clone [11]. The resulting *Sac*I–*Hin*dIII inserts containing the whole coding region of the rat COMT gene and the 5′ end non-coding regions without (clone 1) or with (clone 2) a 27 bp sequence were ligated into the *Sac*I–*Hin*dIII sites of the vector pGEM3 (Promega) (Figure 3b). For the experiments in COS-7 cells, the *Sac*I–*Hin*dIII fragments were blunted and ligated into the blunted *Kpn*I site downstream of the transcription initiation site of the pSVL SV40 expression vector (Pharmacia) in an appropriate orientation.

Plasmid construction for the study of the translation initiation contexts

The construction of clones 3, 4 and 5 (Figure 3b) has been described previously under the names pR-MB2, pR-S1 and pR-MB3 respectively [10].

Point mutations around the MB-ATG codon in clone 6 (Figure 3b) were created by using PCR as follows. The upstream primer was PR7 (Figure 3a, Table 1), changing C at position +4 in clone 5 to G. This primer contains a *Bam*HI site in the 5′ end. The downstream primer was PR6. The *Bam*HI–*Bst*XI fragment

of the PCR product was then ligated into the respective sites in clone 5.

The COMT-specific fragment of clones 3, 4 and 5 (*Bam*HI–*Hin*dIII in clones 3 and 5, and *Hin*dIII in clone 4) was blunted and ligated into the blunted *Kpn*I site of the vector pSVL SV40 to carry out the experiments in COS-7 cells.

In vitro and *in vivo* experiments

In vitro translations of the COMT plasmid constructs were performed with the TNT T7 Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's recommendations. In some experiments, canine pancreatic microsomal membranes (Promega) were added (4 eq./reaction). For pulse–chase experiments the lysates were incubated in the presence of [^{35}S]methionine (30 μCi/25 μl) and then chased with 1 mM (final concentration) non-radioactive methionine for the time period indicated.

In vivo translations were performed using COS-7 cells (African Green monkey kidney cells, ATCC CRL-1651). Cells were cultured as described [18] and transfected with 5 μg of DNA constructs using the lipofectin procedure [19]. After 18 h incubation, cells were labelled with [^{35}S]methionine (40 μCi/ml) in methionine-free medium for the time period indicated. The chase was performed by incubation with 1 mM (final concentration) non-radioactive methionine for the time period indicated. Rat glial tumour C6 cells (ATCC CCL 107) were labelled with [^{35}S]methionine (200 μCi/ml) for 13 h in methionine-free minimal essential medium (Gibco). The chase with 1 mM (final concentration) non-radioactive methionine was performed for the time period indicated. Cells were lysed and equal amounts of proteins were incubated with 1:100 dilution of the polyclonal anti-COMT serum, and precipitated with Protein A–Sepharose (Pharmacia) as described [18].

In vitro and *in vivo* samples were subjected to SDS/PAGE (10% gels), dried and exposed to Kodak X-Omat films. Quantification of the autoradiograms and Western-blot filters was carried out by densitometry (Ultroscan XL; LKB).

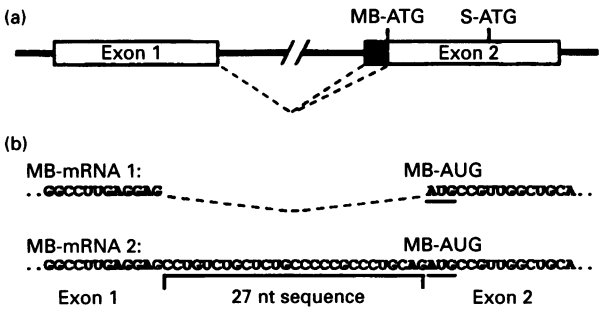

Figure 2 Alternative splicing of non-coding exon 1 to exon 2 in rat COMT MB-mRNA

(**a**) Schematic representation of non-coding exon 1 and the first coding exon 2 of rat COMT gene. Translation initiation codons (MB- and S-ATG) in exon 2 are indicated. The bars between the exons show the alternative splicing events. The black box represents the 27 bp sequence upstream of the MB-ATG codon. (**b**) Sequences of the alternative splicing products, MB-mRNA 1 and MB-mRNA 2, of rat 1.9 kb MB-mRNA. The regions containing the MB-AUG codon, the 27 nt fragment and some adjacent sequences are depicted. The boundaries of the exons and the 27 nt fragment are shown under the sequences. The rest of the COMT sequence is as described previously [13].

RESULTS

Different COMT polypeptides and mRNA size classes in rat tissues

To observe the presence of the COMT polypeptides in selected rat tissues, Western blotting was performed using a polyclonal anti-COMT serum recognizing both COMT forms [6]. A 24 kDa S-COMT and 28 kDa MB-COMT were detected in liver (93% of COMT proteins were in the S-COMT form), kidney (75%), heart (79%), cerebellum (86%) and telencephalon (69%) (Figure 1a). The liver contained high amounts of COMT protein [20–22], confirming previous results.

Northern-blot analysis (Figure 1b) indicates the presence of the two classes of COMT-specific transcripts, with sizes of 1.6 kb (S-mRNA) and 1.9 kb (MB-mRNA). In the liver and kidney most of the mRNA is the shorter species, whereas in the heart and brain tissues the longer mRNA is dominant. PCR was used to confirm the presence of the 1.9 kb mRNA in the liver and kidney. Amplification with primers PR1 and PR2 should give a 637 bp fragment if the 1.9 kb mRNA species is present. As shown in Figure 1(c), this is the case in all the tissues studied.

The 5′ ends of rat 1.6 kb COMT S-mRNA have been shown to reside 17–18 nt upstream of the S-AUG codon [13]. This region also contains putative splicing acceptor sites enabling the formation of longer splicing variants which would have only the S-AUG codon. To detect such mRNAs in rat telencephalon and liver (as a control) poly(A)$^+$ RNAs, they were analysed by the nuclease-protection method using probes NP2 and NP3 [13]. Both of these probes should give a pattern of 70–75 bp-protected fragments if the tissue contains the 1.6 kb S-mRNA and/or putative long splicing forms. The results (Figure 1d) confirm that the liver contains the 1.6 kb S-mRNA in large amounts, and that the S-mRNA and hypothetical long splicing forms containing only the S-AUG codon are either absent from the telencephalon or the quantity is below the detection limit of this sensitive method. Using the same technique, it has been shown previously that the rat heart tissue contains a small amount of 1.6 kb S-mRNA [13]. Thus it can be concluded that the 1.9 kb MB-mRNA is constitutively expressed in different rat tissues at variable levels, whereas the production of the shorter 1.6 kb S-mRNA is tissue-specifically regulated.

MB-mRNAs consist of different splicing variants

The transcriptional initiation and overall splicing pattern of the longer 1.9 kb rat COMT mRNA has been characterized earlier [13]. In order to disclose structural variation in the 5′ non-coding region of the 1.9 kb mRNA in detail, rat heart poly(A)$^+$ RNA was used to prepare cDNA clones because of its high content of 1.9 kb MB-mRNA. A total of 40 separate 5′-specific COMT cDNA clones were isolated. Sequencing of the clones revealed two different splicing variants (Figure 2). One of the forms (MB-mRNA 1) was identical with that described previously [13], where the splicing of the non-coding exon 1 occurs in the splicing acceptor site located immediately upstream of the MB-AUG codon. In the other form (MB-mRNA 2) the splicing occurs in the acceptor site located 27 nt upstream.

The ratios of the two MB-mRNA splicing variants in selected rat tissues were determined using the solid-phase minisequencing technique [23]. The analysis of several different purified rat RNA preparations was performed with primers distinguishing between the two variants. The results indicated that the MB-mRNA 1 form was dominant in all tissues studied (liver, kidney, heart, stomach, duodenum, telencephalon, cerebellum, pons medulla

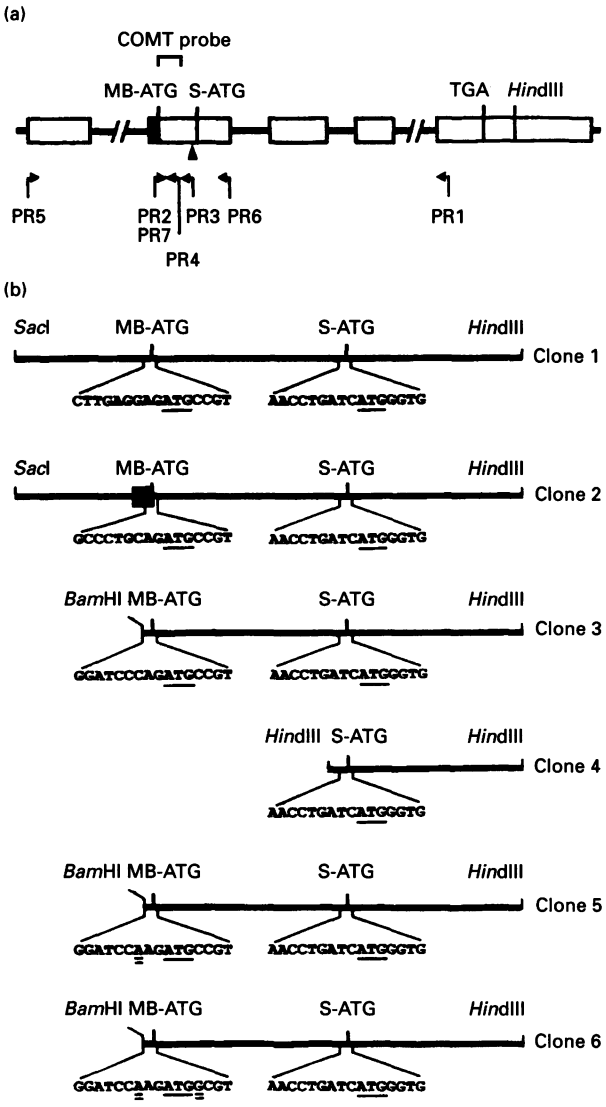

Figure 3 Rat COMT plasmid constructs

(**a**) An overview (not to scale) of the rat COMT gene showing the five exons (boxes), translation initiation codons, translation stop codon (TGA), and an internal *Hind*III site used in clonings. The transcription initiation site of S-mRNA is indicated by an arrowhead. Also the locations of primers used in the tailing reactions, cDNA synthesis, cloning procedures and PCR, and the COMT-specific probe used in the tailing reactions are shown. ■, the 27 bp sequence upstream of the MB-ATG codon. (**b**) COMT fragments used in *in vitro* and *in vivo* experiments (not to scale). Translation initiation codons and restriction sites used in clonings are shown. ■, the 27 bp sequence upstream of the MB-ATG codon. The sequences of the translation initiation regions are indicated below the constructs. The translation start codons are underlined and the mutated nucleotides are double-underlined.

and striatum). The amount of MB-mRNA 2 was always 7% or less of total MB-mRNA.

Effect of the 27 nt sequence and context changes on the translation initiation in the MB-AUG codon

The presence of the 27 nt sequence alters the translation initiation context of the MB-AUG by changing the G at position −3 to C. To analyse whether this alteration affects the initiation of translation from this AUG codon, full-length cDNA constructs

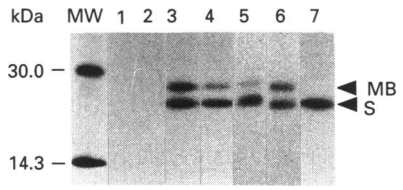

Figure 4 Effect of the context of the MB-AUG codon on translation initiation

Different rat MB-mRNA constructs in pSVL simian virus 40 expression vector were expressed for 18 h in COS-7 cells, immunoprecipitated with anti-COMT serum, and subjected to SDS/PAGE (10% gels) as described in the Materials and methods section. Lane MW, molecular-mass marker; lane 1, pSVL SV40 vector + anti-COMT; lane 2, clone 1 + preimmune serum; lane 3, clone 1 + anti-COMT; lane 4, clone 2 + anti-COMT; lane 5, clone 3 + anti-COMT; lane 6, clone 5 + anti-COMT; lane 7, clone 4 + anti-COMT. The positions of MB- and S-COMT polypeptides are indicated on the right.

of MB-mRNA without (clone 1) or with (clone 2) the 27 bp fragment were prepared (Figure 3b). The 5′ end of both cDNAs is located 160 bp upstream of the MB-ATG codon. This region has previously been determined to contain the major transcription initiation sites of the 1.9 kb transcripts [13].

The translational capacities of the constructs were analysed in transfected monkey COS-7 cells. The results of the immunoprecipitations (Figure 4) show that the efficiency of translation initiation from the MB-AUG codon in these cells is different in the two MB-mRNA splicing variants. In clone 1 (no 27 nt sequence, G at −3), the ratio of the MB- to S-COMT form produced was 0.7 (lane 3) and in clone 2 (27 nt sequence present, C at −3) it was 0.3 (lane 4).

To determine whether the observed differences in the translation initiation was in fact due to the change of the nucleotide at position −3 or the RNA sequence(s) located further upstream of this site, the following two constructs were analysed in COS-7 cells. In clone 3 (Figure 3b) the COMT-specific sequence starts at position −3 (C at this site). Clone 5 contains a point mutation at position −3 (changing C to A) altering the initiation context of MB-AUG to be more favourable [14, 24]. The *in vivo* results (Figure 4) show that the optimizing of the MB-AUG codon context indeed enhances the translation initiation from this codon. The ratio of MB- to S-COMT polypeptide produced is higher (0.2 in clone 3 and 0.6 in clone 5) when MB-AUG is in the more favourable context (Figure 4, lanes 5 and 6 respectively). The ratios of COMT polypeptides produced from these constructs were comparable with those produced from the full-length clones 1 and 2. This suggests that the differences in the efficiency of translation initiation from MB-AUG in the splicing variants is mainly due to the nucleotide change at position −3.

It can also be seen that clone 4 (no MB-ATG codon) produced only S-COMT protein as expected (Figure 4, lane 7).

Determination of the proteolytic processing of MB-COMT polypeptide

To determine if proteolytic cleavage participates in the production of the S-COMT form from MB-COMT, the following pulse–chase experiments were carried out *in vitro*. Clones 1 and 2 (Figure 3b) were translated in rabbit reticulocyte lysates supplemented with canine microsomal membranes. Densitometric scanning showed that the ratio of MB- to S-COMT polypeptide remained unchanged in both clones (3.1 and 1.7 in clone 1 and 2 respectively), indicating that no proteolytic processing of MB-COMT to S-COMT occurred (Figure 5, lanes

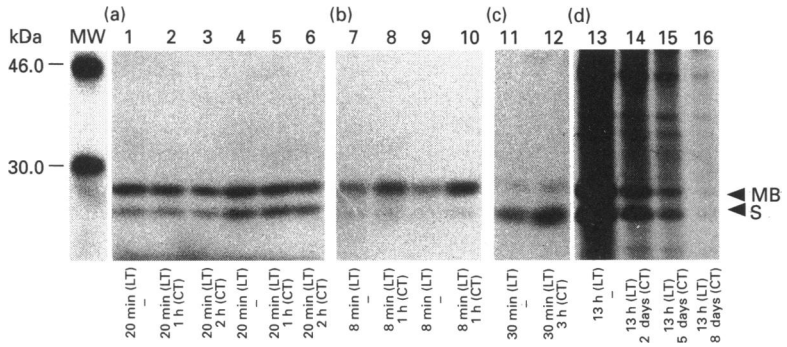

Figure 5 Analyses of proteolytic processing of MB-COMT polypeptide

The labelling (LT) and chasing (CT) time periods are indicated below the lanes. In all cases the samples were subjected to SDS/PAGE. Lane MW, molecular-mass markers. The positions of MB- and S-COMT polypeptides are indicated on the right. (**a**) *In vitro* pulse–chase experiments were carried out using clones 1 and 2. The translations were performed with the TNT T7 Coupled Reticulocyte Lysate System (Promega) supplemented with canine microsomal membranes (4 eq./reaction). Lanes 1–3, clone 1; lanes 4–6, clone 2. (**b**) *In vitro* pulse–chase experiments using clone 6 in either the absence (lanes 7 and 8) or presence (lanes 9 and 10) of canine microsomal membranes. (**c**) *In vivo* assay of clone 3. The expression was carried out in COS-7 cells followed by immunoprecipitation with anti-COMT serum. (**d**) *in vivo* pulse–chase experiments of C6 cells. The cell lysates were immunoprecipitated with anti-COMT serum.

1–6). To investigate whether rapid proteolytic processing after the synthesis of MB-COMT would give rise to S-COMT protein, a pulse–chase experiment was performed *in vitro* using an 8 min pulse and a 60 min chase. Clone 6 (Figure 3b), which contains mutations at positions −3 (changing C to A) and +4 (changing C to G), was used in this experiment. Because of the optimal context of the MB-AUG in clone 6, the predominant translation product would be MB-COMT, thus making the detection of the appearance of the S-COMT protein easier. The results (Figure 5, lanes 7–10) show no difference in the relative amount of S-COMT produced during the pulse or after the chase. A similar result was obtained when canine microsomal membranes were present in the lysate.

To analyse if proteolytic processing occurs *in vivo*, a pulse–chase experiment was performed in COS-7 cells transfected with clone 3 using a labelling time of 30 min followed by 3 h chase. The results show no conversion of MB-COMT into S-COMT (Figure 5, lanes 11 and 12). Furthermore, the *in vivo* pulse–chase studies were performed with rat glial tumour C6 cells expressing only the 1.9 kb MB-mRNA (data not shown) analogously to the rat brain tissues. Cells were labelled for 13 h and then chased for 2, 5 and 8 days. The MB/S ratios varied between 1.1 and 1.4 (Figure 5, lanes 13–16).

Together, the *in vitro* and *in vivo* experiments suggest that proteolytic processing does not significantly contribute to the production of S-COMT protein. Rather, it is expressed from the 1.9 kb MB-mRNAs by the leaky scanning mechanism of translation initiation or from the 1.6 kb S-mRNA. This is supported by the fact that the translation initiation context of MB-AUG in rat is not optimal (C at +4) and thus the codon is able to leak during translation.

DISCUSSION

COMT enzyme occurs in mammalian cells in two forms (soluble and membrane-bound) having different subcellular localizations [3,20,22]. To understand how the two forms of this enzyme are synthesized in rat tissues, we have previously shown that two promoters in the single COMT gene regulate the expression of separate MB- and S-mRNAs [13]. This and our earlier study [13] show that some rat tissues, such as the liver and kidney, contain both COMT mRNA species, whereas some of the tissues, such as the brain, contain only MB-mRNA. Despite this clearcut difference in the COMT mRNA composition between the tissues, the majority of COMT protein in them consists of S-COMT (see Figure 1a). Protein analyses revealed S-COMT as a duplet band in some of the rat tissues studied (liver, telencephalon, cerebellum). Notably, this phenomenon is not observed in the continuous C6 cell line, where only the smaller S-COMT species is detected (results not shown). The reason for the different S-COMT species in different tissues is not clear at present. Here we exclude the possibility that the 1.9 kb mRNA in rat brain contains a hypothetical splicing variant which lacks the MB-AUG, and thus could be responsible for the synthesis of S-COMT polypeptide. Consequently, the MB-mRNA must direct the production of both COMT proteins in tissues such as the brain.

Two mechanisms have been proposed to explain the synthesis of the two COMT polypeptides from one MB-mRNA species: (1) the S-COMT form is initiated from the downstream (S-AUG) start codon in the 1.9 kb MB-mRNA by the leaky scanning mechanism of translation initiation [10,13], or (2) S-COMT polypeptide is produced by post-translational proteolytic processing from the MB form [9,16].

The analysis of COMT MB-mRNA 5′-specific cDNA clones revealed two different mRNA species formed by alternative splicing, MB-mRNA 1 and MB-mRNA 2. Both splicing variants carry MB- and S-AUG codons, but MB-mRNA 2 contains an additional 27 nt sequence upstream of the MB-AUG codon. Notably, this extra sequence alters the translation initiation context of the MB-AUG codon present in MB-mRNA 1 by changing G at position −3 to C in MB-mRNA 2. At position +4 there is C in both variants. The leaky scanning mechanism of translational initiation states that the nucleotide at position −3 is the most critical one for the efficiency of translation initiation [14]. When there is a purine at this position, the rest of the context has only a marginal effect. If it is not a purine, then the nucleotide at +4 should be G for efficient translation. Also other sequence(s) 5′ to the start codon and secondary structure can affect the efficiency of translation initiation from a particular AUG [15].

The efficiency of translation initiation in MB-mRNA 1 and 2, and the effect of upstream non-coding region(s), were analysed in monkey COS-7 cells. The results indicated that the alteration in MB- to S-COMT ratio was due to the change of the nucleotide at position −3, and not due to the sequence(s) further upstream

in mRNAs possibly affecting the translation initiation, for example via a secondary structure. A minor fraction of 1.9 kb mRNA was found to initiate transcription at the P2 promoter approximately 50 nt further upstream [13] bringing a GC-rich region to the 5′ end of the splicing variants. This GC-rich (and hence putatively highly structured) sequence did not affect the ratio of MB- and S-COMT produced (results not shown). Even though alternative splicing in 5′ non-coding regions is not uncommon [25], to our knowledge this is the first case where splicing changes the context and thus the efficiency of the translation initiation from a particular AUG start codon.

MB-mRNA 1 and 2 are formed by choosing between two alternative 3′ splicing acceptor sites which are used at different efficiencies. It is known that elements necessary, but not sufficient, for efficient 3′ splicing include a 3′ acceptor site and a polypyrimidine tract [26]. The downstream 3′ splice site used by MB-mRNA 1 is preceded by a pyrimidine-rich sequence, whereas the region preceding the splice site of MB-mRNA 2 is pyrimidine-poor. The determination of the tissue-specific distribution of 1.9 kb MB-mRNA splicing variants, by applying the solid-phase minisequencing method [23], showed that all tissues studied contained 7% or less of MB-mRNA 2. It remains to be shown whether selection of the splicing acceptor site in the MB-mRNA is used to regulate the balance between S- and MB-COMT proteins, for example in other tissues or during ontogeny.

The putative post-translational proteolysis of MB-COMT to yield S-COMT was studied by expressing constructs that produce different ratios of the two COMT proteins by *in vitro* translation and in transfected COS-7 cells. These experiments indicated no production of S-COMT from MB-COMT. Furthermore, in pulse–chase studies with the rat glioma C6 cell line using chase periods of up to 8 days, the ratios of COMT products stayed at similar levels. This suggested that no slow proteolytic cleavage of MB-COMT to S-COMT occurred and that the half-lives of the S- and MB-COMT forms were similar *in vivo*. Proteolysis was also studied by incubating *in vitro* translation products of clone 3 in the presence of different amounts of rat brain and liver crude tissue homogenates for 18 h at 37 °C. No change in the total amounts of MB- and S-COMT or in the MB/S ratios was observed between the samples or compared with the control incubated without the homogenates (results not shown).

Together the *in vitro* and *in vivo* results suggest that S-COMT is not a cleavage product of the MB-COMT form. Rather, depending on the COMT mRNA composition in a particular tissue, S-COMT is produced by either alternative use of translation initiation codons in MB-mRNAs or translation from S-mRNA, or both. This is further supported by the fact that the MB/S ratios in COS-7 cells transfected with clone 1, and C6 cells and telencephalon tissue containing only the 1.9 kb transcripts, are at comparable levels.

Protein analyses of rat crude tissue homogenates with specific antisera revealed that the relative amount of the MB-COMT form in the rat tissues studied is higher than reported previously based on activity measurements [27]. Recent studies have suggested that the MB-COMT polypeptide is located in the rough endoplasmic reticulum (J. Peränen, T. Karhunen, C. Tilgmann, P. Panula and I. Ulmanen, unpublished work). The fractionation procedures used in the earlier quantifications of COMT proteins may have underestimated the amount of the MB-COMT form.

We are grateful to Dr. Pertti Panula for providing us with the tissue samples, to Ms. Seija Ukkonen and Mrs. Raija Savolainen for excellent technical assistance and to Dr. Carola Tilgmann for assistance in the Western blotting. We thank Dr. Päivi Lehtovaara for critical comments on the manuscript.

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Received 27 May 1993/13 July 1993; accepted 16 July 1993