Vol. 174, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1991 Pages 156-162

THE PRODUCTION BY ALTERNATE SPLICING OF TWO mRNAs DIFFERING BY ONE CODON COULD BE AN INTRINSIC PROPERTY OF NEUROENDOCRINE PROTEIN 7B2 GENE EXPRESSION IN MAN

Luc Paquet, Claude Lazure, Nabil G. Seidah, Michel Chrétien, and Majambu Mbikay

Laboratoires J.A. de Sève de Neuroendocrinologie Moléculaire et Biochimique, Institut de Recherches Cliniques de Montréal, Montréal, Québec, Canada H2W 1R7

Received November 9, 1990

Two types of mRNAs for neuroendocrine protein 7B2 were deduced from the sequence of cDNAs clones isolated from a human pituitary cDNA library. One type lacks an Ala_{100} codon present in the other. The difference is located at an intron site within the human 7B2 gene and can be explained by the transcriptional utilization of two alternate acceptor splice sites, three nucleotides apart. Heteroduplex analysis of DNA fragments amplified by the polymerase chain reaction indicated that this 7B2 mRNA dimorphism occurs in several human endocrine tissues as well as in other species, suggesting that the alternate processing of 7B2 gene transcripts may be an intrinsic mechanism of its expression and could underlie some yet unknown biological functions.

7B2 is an acidic protein of 22 kDa stored within dense-core vesicles in many neuronal and endocrine cells, from which it could be released by various secretory stimuli (1, 2 for a review). The complete primary structure of 7B2 in man, pig, mouse and frog has been elucidated by cDNA cloning and sequencing (2-5). The remarkable conservation of its sequence during evolution, combined with its characteristic tissue distribution and subcellular localization, led to the suggestion that this protein could play a important role in the pathway of regulated secretion in neuroendocrine cells (3, 6). Noticeable in the cDNA-derived sequence of human 7B2 (3), was the absence, at position 100, of an Alanine which we had previously shown to be present by protein sequencing (7). This difference could be attributed either to genetic polymorphism in the human population or to alternate splicing during transcription. Here, we report the isolation of human pituitary cDNAs representative of both types of mRNA; we describe a possible transcriptional mechanism by which they are generated; and we demonstrate that this dual production may be occurring in various human and animal tissues.

0006-291X/91 $1.50

METHODS

Cloning of human pituitary 7B2 cDNAs. Standard molecular biology protocols (8) were applied to screen a λgt10 library of human pituitary cDNAs and to analyse and sequence positive cDNA clones.

Blot Analysis. Southern blot analysis was performed as previously described (9), except for the following modifications: the membrane was hybridized to the probe in 6 x SET (1 x SET = 0.15 M NaCl, 30 mM Tris-HCl, 1 mM EDTA, pH 8) for 16 h at 69°C, and then washed at 50°C either in 2 x SET - 0.1% SDS for 30 min (low stringency) or in 0.1 x SET - 0.1% SDS for 45 min (high stringency). The probe was produced from these two partially complementary oligonucleotides

5'-TGTTGGAAAAACAGA-3' (sense)
3'-TTTTTGTCTACTACCTACAGA-5' (antisense)

by selectively extending the antisense strand to 27 nucleotides (nt) with the Klenow fragment of *E. coli* polymerase I and [^{32}P] dATP and dCTP. This 27-mer probe (P7B2/27) covers the polymorphic region in the human 7B2 sequence lacking codon$_{100}$ (nts 391-417)(3).

Amplification of DNA fragment encoding 7B2. Total RNA was isolated from tissue homogenates in a guanidium isothiocyanate buffer by acid phenol extraction and ethanol precipitation (10). Ten micrograms of RNA were primed with (dT)$_{12-18}$ and reverse-transcribed into cDNA using a kit from InVitrogen (San Diego, CA). After degradation of excess RNA in 0.2 M NaOH for 2 h at 70°C, phenol chloroform extraction and ethanol precipitation, the cDNA was dissolved in 50 μl of 10 mM Tris-HCl/1 mM EDTA pH 8 and 1 μl used for the polymerase chain reaction (PCR)(11). Amplification was performed with the sense primer H13 (CCAGGCCATGAATCTTGTGGGCCCCCAGAGC) and the antisense primer H14 (GGGACAGACTTCTTTGCAACAACATTATCC) or H15 (CCACTTGCCCAAGCCTGGATAGTC) derived from the human cDNA sequence (3). H13 and H14 primers amplify a PCR fragment of 413 bp; H13 and H15 primers, a fragment of 297 bp. Both fragments encompass the polymorphic region. Primers H13 and H14 exhibit, respectively, 87% and 84% nucleotide sequence homology between man and frog (3, 5) and permitted PCR amplification of 7B2 sequences in several species. The reaction mix (100 μl) contained an undetermined amount of DNA, 1 μM of each primer, 200 μM of dNTP and 2.5 units of Taq DNA polymerase in a buffer containing 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, and 0.01% (w/v) gelatin. The PCR were run in a Perkin-Elmer Cetus apparatus through 30 cycles, each comprising a 1-min denaturation step at 94°C, a 1-min annealing step at 65°C (H13 vs H15 primers) or 50°C (H13 vs H14 primers) and a 1-min extension step at 72°C.

Non-denaturing gel electrophoresis. Ten μl of the PCR reaction mixtures were heated at 94°C for 10 minutes to dissociate the two DNA strands; they were then cooled down to room temperature to allow the formation of the DNA duplexes. They were electrophoresed in a 5% non-denaturing polyacrylamide gel (PAGE) at 70 volts for two hours (12). The resolved DNA fragments were visualized under UV after ethidium bromide staining.

RESULTS AND DISCUSSION

Protein sequencing results. In view of the discrepancy between the cDNA-deduced primary sequence of 7B2 and the one we obtained by direct protein sequencing (7), we re-examined the original protein sequencing data for the possible presence of two 7B2 forms in the material purified from human pituitaries. Indeed, the sequence of a Lysobacter-C peptide corresponding to residues 99-128 (7) did contain two chains: the major chain was characterized

by an initial yield of 493 pmol, a repetitive yield of 87 % and, by the first 16 cycles, corresponded to the sequence Thr-**Ala**-Asp-Asp-Gly-X-Leu-Glu-Asn-Thr-Pro-Asp-Thr-Ala-Glu-Phe (X = uncharacterized Cys residue; note the Ala at position 2); the minor chain was identified by the first six cycles to the sequence Thr-Asp-Asp-Gly-X-Leu-Glu and did not contain an Ala at position 2. From the minor chain yield of Asp, Gly, Leu and Glu at cycles 2, 4, 5 and 6, respectively, and that of major chain residues at corresponding positions, it could be deduced that the minor chain represented approximately 33 % of the sequencable material. This result suggested that the Ala_{100}^{+} form of 7B2 was the more prevalent in the pool of human pituitaries.

Isolation of two different cDNAs coding for 7B2. To further confirm this observation, a human pituitary cDNAs library in λgt10 was screened with a mouse cDNA probe (2); seven positive clones were isolated and their cDNA insert analyzed by Southern blot, using the oligodeoxynucleotide probe P7B2/27, specifically designed to attach to the $codon_{100}^{-}$ 7B2 sequence under high-stringency washing. Two out of seven inserts failed to hybridize to this probe (Fig. 1B), suggesting that they may carry the Ala_{100}. This presumption was confirmed by cDNA sequencing as clearly shown in Fig. 2.

Genetic basis of 7B2 mRNA dimorphism. Since the human haploid genome seems to carry only one copy of 7B2 gene located on chromosome 15 (2, 13), the incidence of two forms of 7B2 mRNA could be due to the presence of two prevalent 7B2 alleles in the human population. In an effort to identify Ala^{+} homozygosity, we screened RNA from two dozens individual pituitaries by slot blot analysis with the P7B2/27 probe. They all gave a positive signal (not

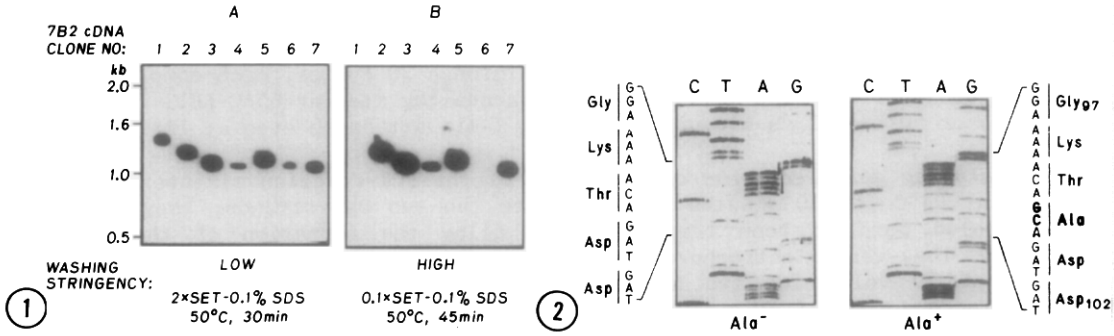

Figure 1. Differential hybridization of human 7B2 cDNA clones. Inserts from clones positive for the mouse 7B2 cDNA probe were excised from phage DNA by EcoRI digestion and analysed by Southern blot using the P7B2/27 probe. The membrane was washed under low stringency condition first, then rewashed under more severe conditions as described in Methods. The autoradiograms shown was obtained between (A) and after (B) the two washings.

Figure 2. DNA sequence ladders in the polymorphic region of human 7B2 cDNA. The cDNA inserts were entirely sequenced. The sequence ladders of clones positive (Ala^{-}) and negative (Ala^{+}) for P7B2/27 probe shows that the latter carries an extra GCA codon specifying an Alanine. No other difference with the published cDNA sequence (3) was observed in the coding region.

shown) suggesting that they all contained $codon_{100}^-$ mRNA. The small number of the analyzed samples notwithstanding, these results hinted at the likelihood that the $codon_{100}^-$ and the $codon_{100}^+$ mRNA might be products of alternate splicing of a single primary transcript.

We have isolated clones containing human 7B2 gene fragments and have determined the sequences at its intron-exon boundaries for most of the gene. The polymorphic site is located at the very start of the third exon. The sequence at the adjoining intron-exon boundary is shown in Fig. 3. Interestingly, there are, in this sequence, two potential acceptor splice sites, three nucleotides apart. Depending on whether the first site or the second site is utilized by the spliceosome, the mature mRNA will contain or lack the codon GCA specifying an Alanine. Although the existence of a GCA mini-exon in the preceding intron cannot be definitely excluded, it is likely that the two mRNAs are generated by alternate splicing at this junction.

Formation of heteroduplexes. To determine if production of the two 7B2 mRNA occurs in other human and animal tissues, we applied the heteroduplex analysis procedure described by Rommens et al (12) for detecting a codon deletion in the cystic fibrosis gene, to 7B2 DNA fragments amplified by PCR from the total cDNA, using primers flanking the $codon_{100}$ region. The two types of cloned cDNAs served as control templates. As shown in Fig. 4A, lanes 1-3, the presence of a slowly migrating heteroduplex form was detected only when

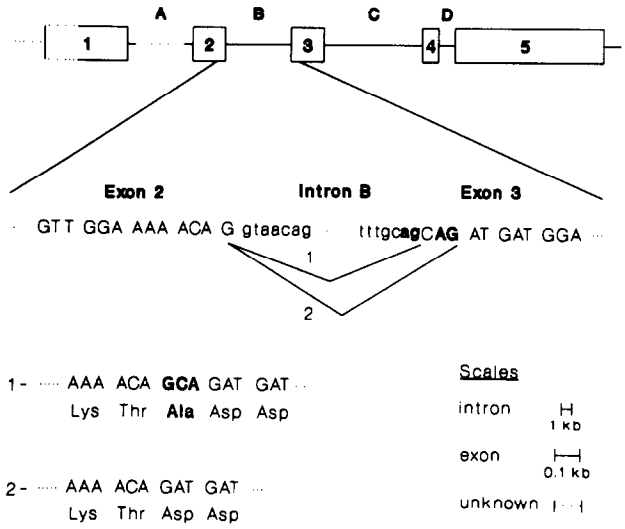

Figure 3. Possible mechanism of biosynthesis of the two 7B2 mRNAs. Partial diagram of the intron-exon organization of the human 7B2 gene derived from overlapping genomic clones: Exons are represented by open boxes. The putative first exon was verified by PCR of genomic DNA using primers derived from the longest cDNA, one located at the 5'-most end, the other just before the first intron site. Its real size as well as that of that of following intron have not yet been determined. The DNA sequence at the boundaries of the second intron the alternate splice sites at the 3' boundary and the resulting mRNA and proteins sequences are shown.

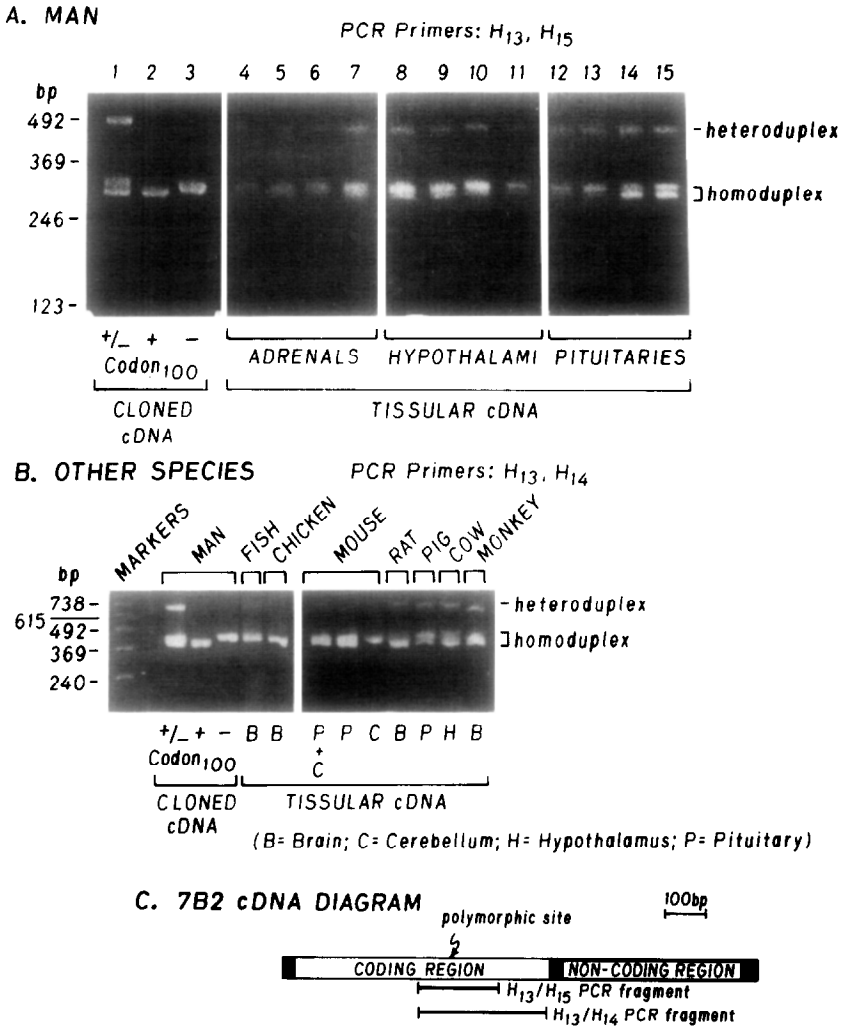

Figure 4. Heteroduplex analysis of PCR fragments of 7B2 in various tissues. The analysis was conducted as described in METHODS. The diagram at the bottom illustrates the relative positions of the amplified fragments along the human cDNA sequence (3).

the products of separate amplifications of the two control templates were mixed, denatured and allowed to anneal prior to PAGE. When endocrine tissues collected post-mortem from different individuals were analyzed by this method, the formation of heteroduplexes of similar migration properties as the mixed controls was observed in all cases (Fig. 4A, lanes 4-15), suggesting that the two forms of 7B2 mRNA forms were expressed in these tissues. By this assay, 7B2 RNAs from endocrine tissues of several (but not all) other species also appeared to be heteromorphic (Fig. 4B).

These results seem to indicate that production of two forms of mRNA is a conserved property of 7B2 gene expression. In the absence of a known biological function for the protein, the physiological relevance of this phenomenon is difficult to assess. By analogy to the case of the insulin

receptor gene, where products of alternate splicing exhibited differential affinity for the hormone (14,15), one can speculate that the relative concentration of the two forms of 7B2 may modulate secretory processes in neuroendocrine cells. Another intriguing observation is the higher frequency of the $codon_{100}^-$ mRNA form among human 7B2 cDNA clones (Fig. 1), in obvious contrast with the prevalence of the Ala_{100}^+ form in the sequence of pituitary-extracted 7B2 protein. In a similar vein, recent data from our laboratory have shown that 7B2 purified from porcine pituitaries contains primarily a Val_{100}^+ form (Lazure et al., manuscript submitted), whereas the cDNA-deduced sequence lacks this residue. It is as though 7B2 gene transcription results in the accumulation of the $codon_{100}^-$ mRNA form which, for translation, is a less favored template than the $codon_{100}^+$ form. This hypothesis will be verified by determining the relative abundance of the two mRNAs on the polysomes from various tissues.

ACKNOWLEDGMENTS: The authors are grateful to Haidy Tadros and Francine Sirois for their technical assistance, to Dr. Olivier Civelli for providing the human pituitary cDNA library. L.P. is a Ph.D. pre-doctoral fellow, and C.L. a scholar of the Fonds de Recherches en Santé du Québec. This work was supported by grants from the Medical Research Council of Canada and La Succession J.A. de Sève.

REFERENCES

1. Seidah, N.G., Hsi, K.L., De Serres, G., Rochemont, J., Hamelin, J., Antakly, T., Cantin, M., and Chrétien, M. (1983) Arch. Biochem. Biophys. 225, 525-534.

2. Mbikay, M., Grant, S.G.N., Sirois, F., Tadros, H., Skowronski, J., Lazure, C., Seidah, N.G., Hanahan, D., and Chrétien, M. (1989) Int. J. Prot. Pept. 33, 39-45.

3. Martens, G.J. (1988) FEBS Lett. 234, 160-164.

4. Brayton, K.A., Aimi, J., Qiu, H., Yazdanparast, R., Ghatei, M.A., Polak, J.M., Bloom, S.R., and Dixon, J.E. (1988) DNA 7, 713-719.

5. Martens, G.J., Bussemakers, M.J.B., Ayoubi, T.A.Y., and Jenks B.G. (1989) Eur. J. Biochem. 181, 75-79.

6. Marcinkiewicz, M., Benjannet, S., Cantin, M., Seidah, N.G., and Chrétien, M. (1986) Brain. Res. 380, 349-356.

7. Leduc, R., Seidah, S., Benjannet, S., Chan, J.S.D., Marcinkiewicz, M., Lazure, C., and Chrétien, M. (1987) In Proteins: Structure and Function (J. L'Italien, Ed.), pp. 789-796. Plenum Press, New York, N.Y.

8. Sambrook, K., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.

9. Mbikay, M., Linard, C.G., Sirois, F., Lazure, C., Seidah, N.G., and Chrétien, M. (1988) Cell. Mol. Biol. 34, 387-398.10.

10. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.

11. Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., and Arnheim N. (1985) Science 230, 1350-1354.

12. Rommens, J., Kerem, B.S., Greer, W., Chang, P., Tsui, L.C., and Ray, P. (1990) Am. J. Hum. Genet., 46, 395-396.

13. Mattei, M.G., Mbikay, M., Sylla, B.S., Lenoir, G., Mattei, J.F., Seidah, N.G., and Chrétien, M. (1990) Genomics 6, 436-440.

14. Goldstein, B.J., and Dudley, A.L. (1990) Mol. Endocrinology, 4, 235-244.

15.. Mosthaf, L., Grako, K., Dull, T.J., Coussens, L., Ullrich, A., McClain, D.A. (1990) EMBO J. 9, 2409-2413.