

# Human cytochrome $b_{561}$ : a revised hypothesis for conformation in membranes which reconciles sequence and functional information

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Cytochrome  $b_{561}$  is a major transmembrane protein of catecholamine and neuropeptide secretory vesicles. In this report, we describe the cloning and properties of a full-length cDNA encoding human neuroendocrine cytochrome  $b_{561}$  from a human caudate cDNA library and a human peripheral blood genomic library. The human cDNA contains two major transcription start sites and only one translation start site that codes for an apocytochrome  $b_{561}$ , which is 22 amino acid residues smaller than the previously deduced amino acid sequence from bovine

cDNA. This smaller version of cytochrome  $b_{561}$  may contain only five transmembrane segments rather than the previously proposed six segments. The new model is in agreement with our previous results on transmembrane topology of the gene product. Northern-blot analysis shows an expanded tissue distribution of cytochrome mRNA expression where previous immunological assays were negative. These results support the hypothesis that cytochrome  $b_{561}$  is a marker for peptidergic and adrenergic tissues.

## INTRODUCTION

Cytochrome  $b_{561}$  is present in the membranes of chromaffin granules and other neuroendocrine secretory vesicles (Flatmark and Terland, 1971). This cytochrome functions as a unique transmembrane electron transfer protein by mediating the transfer of electrons from a soluble cytoplasmic donor (ascorbate) across a membrane bilayer to a soluble intravesicular acceptor (semidehydroascorbate) (Njus et al., 1983; Srivastava et al., 1984; Wakefield et al., 1986b). This transmembrane electron transfer activity effectively regenerates the ascorbic acid inside neuroendocrine secretory vesicles, thereby providing reducing equivalents for intravesicular dopamine  $\beta$ -hydroxylase and peptidyl  $\alpha$ -amidating mono-oxygenase (Beers et al., 1986; Menniti et al., 1986; Wakefield et al., 1986a; Kent and Fleming, 1987; Dhariwal et al., 1989). Thus cytochrome  $b_{561}$  acts as a transmembrane electron carrier which catalyses a long-range electron transfer between two cellular compartments.

This long-range electron transfer is an integral part of most neuroendocrine secretory vesicle membranes and it is necessary to understand the structure and function of cytochrome  $b_{561}$  in order to investigate this process in detail. The primary structure of bovine cytochrome  $b_{561}$  has been predicted from cDNA to be 273 residues in length and a model has been proposed in which the polypeptide spans the membrane six times and contains little extramembranous sequence. Since the cytochrome does not contain a cleavable signal peptide, this model predicts an orientation of the protein in which both the N-terminus and C-terminus would be localized to the cytoplasmic side of the secretory vesicle membrane ( $N_{\text{cyt.}}$ - $C_{\text{cyt.}}$ ) (Perin et al., 1988). However, although topological studies have demonstrated that the C-terminus is exposed to the cytoplasmic side of the secretory vesicle membrane, the N-terminus is not facing the cytoplasmic side as it is unavailable to antibodies or proteases on that side of the membrane (Kent and Fleming, 1990). Thus the proposed model is not completely supported by the available experimental evidence.

With regards to its function, cytochrome  $b_{561}$  fulfils the important role of regenerating intravesicular ascorbate for use by the vesicular mono-oxygenases. Therefore, it has been proposed that cytochrome  $b_{561}$  may be a marker for neuroendocrine secretory vesicles (Perin et al., 1988). Yet evidence to date has suggested that cytochrome  $b_{561}$  is not present in all of the expected tissues. This apparent paradox raises the question of the *in vivo* requirement for cytochrome  $b_{561}$  in neuroendocrine biosynthetic pathways.

In as much as structural and functional data from bovine cytochrome  $b_{561}$  has proved difficult to reconcile, we have now turned our attention to cytochrome from human brain. We anticipate that new information from this approach will answer questions raised by previous investigations with the bovine species, as well as allow future studies related to the wealth of functional data available from human brain. We report here the cloning of the human cytochrome  $b_{561}$  cDNA and the detection and expression of cytochrome mRNA in various human tissues. Our results suggest that the structure and distribution of cytochrome  $b_{561}$  is different from previous interpretations based on the bovine data.

## EXPERIMENTAL

### Isolation of partial cDNA clones for cytochrome $b_{561}$

A human caudate cDNA library in  $\lambda$ gt10 (Stratagene) was screened with a cDNA probe encoding the open reading frame of the bovine cytochrome  $b_{561}$  cDNA clone, pcyt6a (base pairs 91–912). This probe was generated by PCR amplification of bovine cytochrome  $b_{561}$  cDNA as template with a sense primer starting with ATG (5'-ATGTGGAGGACCTTGACTTG-CACC-3') and an antisense primer (5'-TCACTGGGAGCT-GGGGCTGTCCCTCCG-3') ending with stop codon. The PCR product was obtained by initial denaturation of the template at 95 °C for 2 min, followed by 35 cycles of: 95 °C, 1 min denaturation; 55 °C, 2 min annealing; and 70 °C, 3 min extension. The final extension was carried out at 70 °C for 7 min.

The PCR product was purified using a gene cleaning kit (Bio-101) and labelled with  $^{32}\text{P}$  by nick translation (Gibco, BRL). The clone pcyt6a was a kind gift from Dr. T. Sudhof (University of Texas, TX, U.S.A.). Plaque hybridization was carried out at 37 °C for 16 h in a solution containing  $6 \times \text{SSC}$  ( $1 \times \text{SSC}$ : 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 1.5% (w/v) SDS,  $5 \times$  Denhardt's solution [ $1 \times$  Denhardt's solution: 0.02% BSA, 0.02% Ficoll, 0.02% poly(vinylpyrrolidone)], 50% formamide, and 100  $\mu\text{g}/\text{ml}$  denatured salmon sperm DNA. Two positive clones (HCR1a and HCR1b) were identified and three rounds of screening were carried out to obtain the pure clones. The  $\lambda$  DNA was prepared using Qiagen lambda kit according to the manufacturer's instructions. The *Eco*R1 fragments were purified and subcloned into the plasmid pGX2627 (generously provided by Genex Corp.).

### DNA sequencing and analysis

Plasmids containing the DNA inserts were digested with several restriction enzymes to obtain smaller size fragments convenient for DNA-sequence analysis. Restriction fragments were incorporated into the pGX 2627 vector which was cut with the appropriate restriction enzymes. Double-stranded DNA sequencing was performed by the dideoxy method using primers from the vector sequences or synthetic oligonucleotides synthesized on the basis of available sequence information. The entire DNA sequence reported was derived from sequencing each strand of the inserts at least once. Sequencing reagents were obtained from U.S. Biochemicals Corp. (Sequenase version 1.0). DNA sequence analysis was performed using the MacVector software package from International Biotechnologies, New Haven, CT, U.S.A. Protein-sequence analysis was performed using the University of Wisconsin Genetics Computer Group Sequence Analysis software package.

### Isolation of full-length human cytochrome $b_{561}$ cDNA

The cDNA clones obtained did not contain the complete coding sequence. Therefore, a PCR fragment was generated as described before using the partial human cytochrome  $b_{561}$  cDNA as template. The primers used were HCPI (422–446, see Figure 1: GGCAGCCACCCACAGCACTGCCT) and HCRP1 (1160–1138, see Figure 1: TCAGTGGGAGCCGGGGCTA-TCTC) from the coding region of HCR1a. The PCR product was purified by gene cleaning and was used to screen a human brain cDNA library (Clontech). The three cDNA clones isolated were sequenced and they contained no further 5' sequences. Therefore, a human peripheral blood genomic library in lambda gem was screened with the above-mentioned PCR probe from human cytochrome cDNA. Plaque hybridization was carried out using the conditions described before except that the temperature was 42 °C. Four positive clones were identified and were plaque purified by sequential plating. The  $\lambda$  DNA prepared from these clones were subjected to restriction enzyme digestion, blotted on to nitrocellulose membrane and probed with a synthetic, antisense oligonucleotide corresponding to the 5' end of the partial cDNA clone, HCASP-1 (469–428, see Figure 1): 5'-AGCTGGGAGA-AGGCCACGTAGTAAGGCAGTGTGTGGGGTG-3' in order to obtain the DNA fragment corresponding to the 5' upstream sequences. One genomic fragment which hybridized to the antisense probe was selected for further characterization.

### Primer extension

Primer extension was carried out using two synthetic antisense oligonucleotides, HCASP-1 (described above) and HCASP-2

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1  CGCATCTCAGGCGCAGTCTCTAGGGGCTGTGCCATCTCAGGGGGGACATGTGCATCTC 60
61  AGGGGGGCTGCTCCATCTGGGGGGTGTGTGTGCATCTCGGGGGGGCTGTGCATCTAGC 120
121  GGGGGTGTGTGTCATCTGGAGGGGGCTGTGCCAACCCGGGGGGGTGTTGCCGGC 180
181  ATCTAGCAGGGGGCGGCTGTGCCATCTCGGGGGGGCTGTGCATCTCGGGGGGACCGT 240
241  GCTTATCTCTGGGGCGGCTGTGCCATCTTAGGGGGTGTGTACATCTCGGGGGGCTGT 300
301  CGCATCTTGGGGGGCTGTGTGCATCCGGGGGGCTGTGCCATCTCGGGGTGCTGTGCC 360
361  TGCTCCTCTGAGCTCTGCTCTTCTTGCAGCGTTCGCTCAGCCATGGAGGGCGGGGCC 420
421  CGGAGCCACCCACAGCACTGCCTTACTACGGTTCCTCCAGCTGCTGGCCCTGA 480
481  CTTTGGTGGCCATGACCGGGCGTGGCTGTACCGAGGGCGGCATTGCCGGGGA 540
541  GCGACCTGCAGTTCAACGGCCACCCCTGCATGGTGCATAGCCCTGATCTTCTCAG 600
601  GAATGCTGCTGGTGTACCTGCTTTCAGGAACGAAGCTAAACGCCACCCAAAGGCTC 660
661  TGACGGGCTGCTGCACATCTTGGCGCTGTACCGCTGGTGGCTTGTGTGGCGGTG 720
721  TGACTACCCAGGAAGAAGGGCTACGCTGACCTGTACAGCTACACAGCTGGTGGGA 780
781  TCCTTGTCTTGTCTGCTACTTGTGCGAGTGGCTGGTGGCTTCCAGCTTCTTCTG 840
841  CCGGAGCTTCACTTCCCTGCGGAGCCCTACCGCCACAGCAGCATCTTCTTGGTCTA 900
901  CCATCTTCTCCCTCCCTGGCCAGCCCTGCTGGCCCTGAAGGAGCAGCTGCTGTCA 960
961  ACCTGGGGGCAAGTATAGCCGATTTGAGCCGAGGGTGTCTGGCCACAGCTGTGGGG 1020
1021  TGCTGCTGGCTGCTTCCGGTGGGGGGTCTACATCTTGACCCGGGGCCAGTGAAGC 1080
1141  ATAGCCCGGCTCCAGTGTAGCGCCGGCCCTGGGGGCTGGGGGCTGCTCTCTD 1200
1201  GCTTGGCCCTGCTGAGCGCTTTCAGGACTGCAGGCTCCGGAGAGTGGCTGTGCCAGC 1260
1261  CGGGGGGGTGGTGCAGGGGGATCCGTTTGTATGCTGCTTCTGGGGCAGGCTCCGCC 1320
1321  TCCTGTGCTTCTCGTTTCTCCGCTGTATAGACAGTTCATTTGTGTGGCTCCCGTGT 1380
1381  TCTGTTGGCCCTTCAAGTGCAGAGGCTTTGGGTAGACTCGGGTGTTCGGTCTGGTGC 1440
1441  CAGGACACAGATCTTAAAGAACCGTTAGAGAGGAGGTTTCTACCCCTTCTGTAGTAA 1500
1501  GCCTGGGGCAAGCCAGGGGAACTGGGGGGCTCAGGGACAGGCTGGAAGGCCAC 1560
1561  GATGGCCTGCTGAATCAAAACAGGAGTCCCTCCAGCCTGAATAACAGTGGCACAAAT 1620
1621  GGGCCGGCTTGGCAGAGGAGCAAGTATGATGTGTAAGATATGTTGGTGGTGAAG 1680
1681  CAAGGTTCCCCAGGAGAGGGGAGGGGCTGCCCTGGGAAGCTCGAGATGAGGCTGG 1740
1741  CCGAGCTGTAGTCTGACCTTACTCTTCTTAAACCCCTTTAGCCCTTAGAGTGGCTGG 1800
1801  TGGGAGAGGGGATAGAAGCCATGACTTCAGACAGACTTCTCTTGGCAGATGCAAGCAG 1860
1861  GCCTCCTCCAGGCTGCTCCAGACATGGGGTGTGGGATGGGGGTACCTTGCAGCCCT 1920
1921  TCCTGCTGGGGCTCCCTCCTTGTAGCACCCCTTGGGGCTCAGCTGTGTTCTCTCCC 1980
1981  AGGCTCACCCAGGCTGCTCAGGCTGGGAGGAGGGCCACAAACCTTATAATTTTTTA 2040
2041  AATGAAAAACCGCTGCTGTGGCTGTGGCTAGAGCCCTTGGGGCTGCTGGAGCTGCTG 2100
2101  CTCTGTCTGGAGGACGAGCCCTTCTCCTTATCTAGTCCGACCTTTCCAGGAAGTCAG 2160
2161  TGGAGTCAGACAACATACGATCATCCCCCGTGGTGTGTCACATCACTCCAGCCCAATA 2220
2221  AGAGTGTGATGTTAGCTGAGTACCATTGTGGCTTCGGCCGGAAATAGTGTGTAAGACA 2280
2281  CTGATCGTGTGCGAGGCCAGGAGATCAAGACCATCTGACTAACAAACAGATGAAACCC 2340
2341  CTGCTCTACCGAATTCCTGTATTAGTCTATATGGTTCTCCAAGAAACTGAATATCCA 2400
2401  TTGGGAAGCGGTGGATAAAGTACGACCAAAATTTGAGAATACATAAACAAGCATCC 2460
2461  GCAGGAACATACAGAGGATGCCTTTTCTGTGATTTGGTGGGATTTTTTCCCTTTTATG 2520
2521  TGGATATAGTAGTTACTTGTGCAAGAATAATTTGGAATAATTTCTATTAATCAACT 2580
2581  CTGAAGCTAATGTACATAAATCTCGAGATGTGTTGTTTCATATAAAGTGAAGTGAAT 2640
2641  CTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 2700

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Figure 1 Human cytochrome  $b_{561}$  cDNA

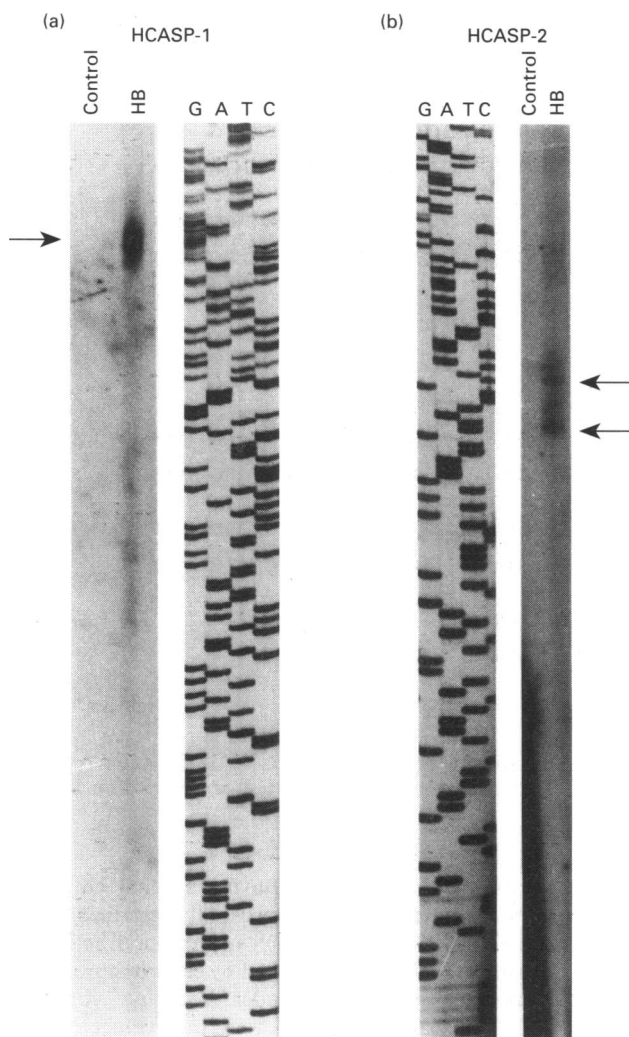
Nucleotide and deduced amino acid sequence of the human cytochrome  $b_{561}$  cDNA. The two major transcription start sites are marked by asterisks and the underlined regions indicate the following: 171–189, PCR primer HCSP-5; 255–273, PCR primer HCSP-4; 428–471, PCR primer HCP1; 593–570, antisense primer HCASP-191; 1160–1135, antisense primer HCRP1; 1618–1748, 3' non-coding region shown in Figure 2b; 2623–2629, the putative poly(A) adenylation site.

(199–171, see Figure 1: 5'-ACAGCCGCCCTGCTAGAT-GCGCGCAACA-3'). The oligonucleotides were end-labelled with  $^{32}\text{P}$ , hybridized to 50  $\mu\text{g}$  of human brain total RNA (Clontech) and extended using murine-leukaemia-virus reverse transcriptase (MLV-RT, Gibco-BRL) for 1 h at 42 °C. Yeast tRNA was used as a negative control. The primer-extended products were separated on an 8 M urea/6% polyacrylamide gel and then visualized by autoradiography.

### Reverse transcription-PCR (RT-PCR)

The RT-PCRs were performed with human brain total RNA according to the manufacturer's instructions (Perkin-Elmer





**Figure 3** Primer extensions

Primer extension of 50  $\mu$ g of total RNA isolated from human brain were performed using oligonucleotides HCASP-1 (a, lane HB), and HCASP-2 (b, lane HB). Yeast tRNA (50  $\mu$ g) was used as negative control (a and b, Control lanes). The primer-extended products were separated on an 8 M urea/6% polyacrylamide gel. The arrows indicate the respective products. Marker lanes G, A, T and C indicates the sequencing ladder of M13 plasmid with universal primer.

6.5 kb *Xba*I genomic fragment from one clone which hybridized was selected for subcloning and sequencing. Examination of the sequences upstream from the region complementary to the 5' end of HCR1a revealed the presence of a translation initiation codon sequence.

#### Analysis of human cytochrome *b*<sub>561</sub> sequence

The sequence information derived from cDNA clones and genomic DNA clones, together with the deduced amino acid sequence, of human cytochrome *b*<sub>561</sub> is shown in Figure 1. The longest open reading frame (bp 405–1157) shows 86% identity with the corresponding sequence of bovine cytochrome *b*<sub>561</sub>. A single translation initiation start site for this open reading frame is found at position 405. This translation initiation site is found in a frequently occurring sequence context for initiation of

translation according to the rules derived by Kozak (Kozak, 1986).

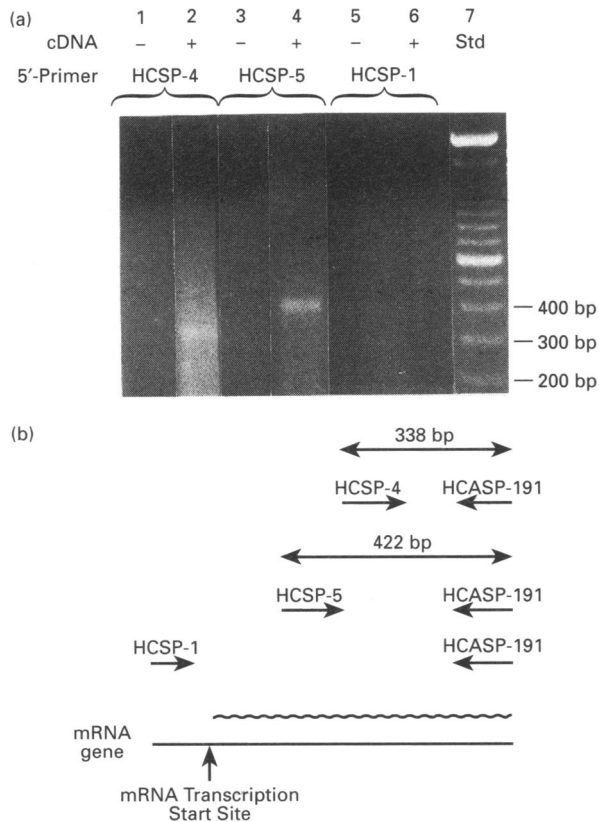
The 5' untranslated region of the human cytochrome *b*<sub>561</sub> mRNA is 404 bases in length. As shown in Figure 2(a), comparison of this region with the corresponding region in the bovine cDNA reveals little sequence similarity. In order to ensure that 5' upstream sequences derived from the genomic clone are present in mRNA, we performed primer extension to assess the size of the mRNA transcript and RT-PCR to obtain the sequence for this region directly from mRNA. Primer extension was carried out using the primer HCASP-1 which is downstream from ATG and another primer HCASP-2 which is in the 5' untranslated region. The former produced a primer extended product of approx. 460 bp (Figure 3a) and the latter produced two major products of approx. 199 and 191 bp with evidence of two other products of similar size (Figure 3b). The major transcription start sites were therefore assigned to the cytosine residues 405 bp and 397 bp upstream from the translation initiation codon (nucleotides 1 and 8 respectively, see Figure 1). The negative control with yeast tRNA did not give any signal with these two primers.

To confirm further that the 5' sequences were not derived from introns, we performed RT-PCR of the human cytochrome *b*<sub>561</sub> RNA. Total RNA from human brain was reverse transcribed into the first-strand cDNA using the antisense 3' primer, HCASP-191. The reaction was then subjected to PCR using the same 3' antisense primer and one of two 5' sense primers: HCASP-4 or HCASP-5. The results are shown in Figure 4. In both cases a single DNA fragment of the expected size (338 bp or 422 bp respectively) was amplified. The presence of potentially contaminating genomic DNA was excluded as no product was obtained in any case if the reverse transcription step was omitted (control lanes). As a final control, we did the PCR experiment using a sense primer (HCASP-1) which hybridizes to the genomic sequences upstream to the transcription start site. This experiment did not give any product in PCR amplifications (Figure 4). The sequence of the 338 bp and the 422 bp PCR fragments derived from mRNA revealed that they were identical with the sequences obtained from the genomic clone. Thus the results of the primer extension/RT-PCR experiment together with the sequence information from mRNA confirm that the 5' untranslated sequences for the human cytochrome *b*<sub>561</sub> are not derived from intron sequences.

The 3' untranslated region of human cytochrome *b*<sub>561</sub> mRNA is 1500 bases in length. A consensus sequence for polyadenylation is found at position 2633. This is shown in Figure 1. A comparison of the 3' untranslated region of the human cDNA to the corresponding segment of the bovine cDNA revealed that most of the sequences had no similarity except for the 130 bp segment of the human cDNA (bp 1618–1748) which is approx. 82% identical with the bovine cDNA (bp 1314–1440). This aligned comparison is shown in Figure 2(b). A consensus sequence from these two regions was constructed and used to search the current nucleotide-sequence databases. No significantly similar sequences were identified in this search. Also, there are no similar open reading frames in this region. We hypothesize that this region may be involved in regulation of cytochrome *b*<sub>561</sub> expression.

#### Analysis of human cytochrome *b*<sub>561</sub> deduced amino acid sequence

The deduced amino acid sequence of human cytochrome *b*<sub>561</sub> predicts a polypeptide of 251 residues encoding a protein of 27.6 kDa. Since the size of the human cytochrome has never been described, we performed a Western-blot analysis of human lung tissue using rabbit anti-(bovine cytochrome *b*<sub>561</sub>) serum. A major



**Figure 4 Reverse transcription PCR**

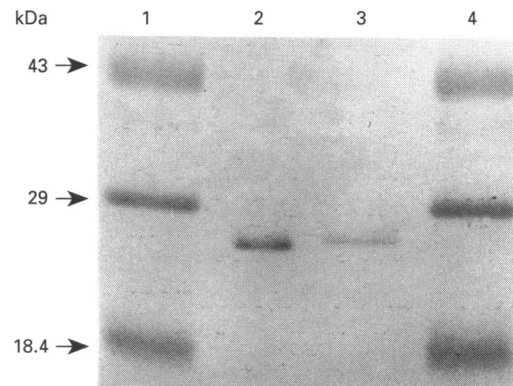
(a) Total RNA isolated from human brain was reverse transcribed into cDNA with HCASP-191 as a specific, 3' antisense primer for human cytochrome mRNA. The cDNA was subjected to amplification by PCR with the addition of one of the following sense primers: HCSP-4 (lane 2), HCSP-5 (lane 4) or HCSP-1 (lane 6). PCR products were analysed by 3.0% agarose gel electrophoresis and visualized by ethidium bromide staining. Negative control for each set of primers are carried out without the reverse transcriptase step (lanes 1, 3 and 5). A 1 kb ladder was used for size markers (lane 7). (b) Schematic representation of the position of primers used for RT-PCR. The size of the expected fragment for each set of primers is shown.

immunoreactive band migrating at an approximate molecular mass of 28 kDa was observed (Figure 5, lane 3). The presence of cytochrome  $b_{561}$  in lung is not surprising in view of the fact that the small granule cells in this tissue contain catecholamine secretory vesicles (Sorokin and Hoyt, 1989), although a larger amount of membrane fraction was needed for the detection of cytochrome  $b_{561}$  compared with chromaffin granule membranes.

A comparison of the amino acid sequences of the human and bovine proteins is shown in Figure 6. The sequences are 86% identical. The human sequence has a residue between positions 8 and 9 of the bovine protein, however, the rest of the sequence is in frame. We have previously postulated that the histidines 109 and 182 of the bovine protein are important for haem binding (Fleming and Kent, 1991). The conservation of these histidine residues in both species supports our hypothesis (Figure 6).

#### Tissue distribution of cytochrome $b_{561}$ expression

As it has been proposed that cytochrome  $b_{561}$  may be a marker for neuroendocrine secretory vesicles, Northern-blot analysis was performed to determine the expression of the mRNA in various human tissues. The results of the duplicate experiment are shown in Figure 7. A major band at approx. 3300 bp was



**Figure 5 Western-blot analysis of human lung tissue**

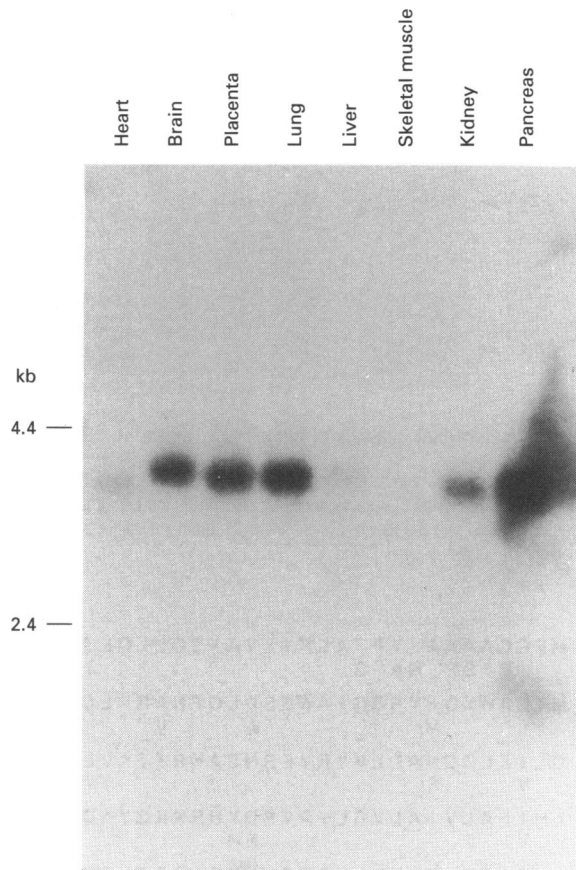
A 0.1  $\mu$ g sample of bovine adrenal chromaffin granule membranes (lane 2) and 60  $\mu$ g of human lung homogenate (lane 3) were separated by SDS/PAGE, transferred to a nitrocellulose membrane, and probed with anti-(bovine cytochrome  $b_{561}$ ) serum. Prestained protein molecular-mass markers are shown in lanes 1 and 4.

1	MEGGAAA	TPTALPYV	VAFS	QLLGL	TLVA	29
	P	SP	RA	G	IV	
30	MTGAWL	GLYRGG	IAWES	DLOF	NAHPL	CMVI
		M		A	V	I
60	GLIFLQ	GNALLV	YRVFR	NEAKR	TTKVL	HGL
	V	P				
90	LHIFAL	VIALV	GLVAV	FDYHR	KKGYA	DLYS
	V	F		EH		
120	LHSWCG	ILVFV	LYFVQ	WL	VGFS	FLLPFGAS
		A	F	A		
150	FSLRSR	YRPQH	I	FFGAT	I	FLLPVGTALLGL
		V		A	S	A
180	KEALLF	NLGGK	YSAF	EP	EGVLAN	VLGLLLA
		E	T	M		
210	CFGGA	VLYI	LTRAD	WKRP	SQAEE	QALSMDF
	T	ATVI			L	
240	KTLRQ	GDSP	GSQ	*	251	
	TE		S			

**Figure 6 Comparison of human and bovine amino acid sequences**

The deduced amino acid sequence of the human cytochrome  $b_{561}$  is shown as a continuous sequence. In those positions where the bovine sequence differs, the alternative bovine residue is indicated by the one letter code below the human sequence. Residues proposed to be part of a haem-binding pocket (Fleming and Kent, 1991) are underlined.

detected in brain, placenta, lung and pancreas, with a moderate amount seen in kidney. The mRNA was not detected in skeletal muscle, with very weak hybridization signals in heart and liver. The blot was stripped off from the cytochrome  $b_{561}$  probe and reprobbed with glyceraldehyde 3-phosphate cDNA to confirm equal amounts of mRNA were present in each lane (results not shown). The distribution of cytochrome  $b_{561}$  in brain agrees with that of peptidyl  $\alpha$  amidase. Although certain areas of pituitary and hypothalamus have high levels of peptidyl  $\alpha$  amidase, the rest of the brain appears to have less mRNA than lung (Brass et al., 1989). A surprising result was the high level of cytochrome mRNA in placenta and pancreas as peptidyl  $\alpha$ -amidating monooxygenase mRNA levels are very low in this tissue in the rat (Brass et al., 1989). These results suggest that it would be worthwhile to investigate the level of dopamine  $\beta$ -hydroxylase mRNA in placenta and pancreas.



**Figure 7** Northern-blot analysis of mRNA from different human tissues

Poly(A) RNA (5 µg) isolated from various tissues was separated on a 1% denaturing agarose gel, transferred to a nylon membrane and hybridized with a 727 bp probe generated from human cytochrome  $b_{561}$  cDNA. Size markers are indicated at the left-hand side. Different tissues are labelled above their respective lanes.

## DISCUSSION

The apocytochrome N-terminus begins with the amino acid sequence MEG for human cytochrome  $b_{561}$ . This sequence corresponds to the second translation initiation site in the bovine cDNA. Analysis of the sequence context of both first and second ATG revealed that the second bovine translation initiation site agrees more closely with the consensus sequence for initiator methionines than does the first translation initiation site (Perin et al., 1988). Western-blot analysis of human lung reveals an immunoreactive band migrating at an apparent molecular mass similar to that found in bovine chromaffin granules. This apparent molecular mass of the bovine cytochrome is most consistent with the second translation initiation site being used. Also, a *Xenopus* clone sequenced at the 5'-end starts with the second translation initiation of the bovine and has a stop codon five amino acids upstream of the ATG (M. Srivastava, unpublished work). Taken together with the results on human cytochrome  $b_{561}$  presented here, these results suggest that bovine apocytochrome is 22 amino acid residues shorter than previously proposed (Perin et al., 1988).

Because the N-terminus of bovine cytochrome  $b_{561}$  cannot be experimentally detected on the cytoplasmic side, sequence from both the human and bovine cytochrome of similar size were

compared and critically analysed in an attempt to create a structural model in which the N-terminus would be located internal to the granule. The most N-terminal membrane-spanning segments were analysed according to the method of Hartman et al. (1989). Importantly, the amino acid sequence in the human cytochrome N-terminal from the first hydrophobic segment has a net charge of zero. Therefore, an  $N_{\text{exo.}}-C_{\text{cyt.}}$  (N-terminus towards the matrix side and C-terminus towards the cytoplasmic side) orientation of the first hydrophobic transmembrane segment is possible according to correlative and experimental results on other signal/anchor segments (Hartman et al., 1989; Parks and Lamb, 1991). Such an orientation would explain the inability of both antibodies and Pronase to interact with the N-terminal region of cytochrome in intact chromaffin granules (Kent and Fleming, 1990).

An  $N_{\text{exo.}}-C_{\text{cyt.}}$  orientation of the first hydrophobic transmembrane segment in cytochrome  $b_{561}$  would require an odd number of transmembrane segments because the C-terminus faces the cytoplasm (Kent and Fleming, 1990). In fact, the second putative transmembrane segment in the original model (Perin et al., 1988) does not meet the conservative criteria for hydrophobicity of transmembrane segments defined by von Heijne (von Heijne and Gravel, 1988) (i.e. a mean 19-residue hydrophobicity > 1.5 on the Engelman–Steitz scale). Furthermore this segment, for both the human and bovine cytochromes, contains several amino acid residues (C, D, N and Q) which have very low abundance in eukaryotic transmembrane segments (von Heijne and Gavel, 1988).

Considering the experimental data on haem content and topological accessibility, together with the predictive criteria on hydrophobic transmembrane segments with low hydrophobicity and the unusual amino acid composition of segment two, a likely model for human cytochrome  $b_{561}$  is shown in Figure 8. In contrast with previous models of the cytochrome, this cytochrome contains five hydrophobic transmembrane segments, an  $N_{\text{exo.}}-C_{\text{cyt.}}$  orientation of the first transmembrane segment and a single haem-binding pocket. These three characteristics are more in agreement with the experimental evidence than previous models based on the bovine sequence. The model also leads to the testable hypothesis that antibodies to the first cytoplasmic loop (Arg-39–Lys-84) should interact with the cytochrome in intact chromaffin granules.

The expression of cytochrome  $b_{561}$  appears to be constitutive when compared with other secretory vesicle protein components which are under hormonal and neuroendocrine regulation (Winkler et al., 1990). The cytochrome also shows distinct tissue-specific expression. This has led to the hypothesis that cytochrome  $b_{561}$  would be a basic feature of all peptidergic and adrenergic tissues. In mammals other than humans, immunological studies have shown that it is present in splenic-nerve terminals and posterior and anterior hypophysis (Hortnagle et al., 1973); in many areas of the brain (Duong and Fleming, 1984); in blood vessels, retina, enteric-nerve fibres, and atrial heart (Pruss and Shepard, 1987); and in thyroid parafollicular cells (Weiler et al., 1989). Curiously no cross-reacting material was observed in bovine pancreas or kidney where it would be expected to participate in the formation of amidated neuroendocrine peptides (Brass et al., 1989). RNA blotting experiments have shown a similar distribution in bovine tissues except that no hybridization was seen in heart ventricles and the data are not available for placenta, pancreas or kidney (Perin et al., 1988).

The expression of cytochrome has not been characterized in humans. The RNA hybridization results reported here show that cytochrome mRNA is also expressed in pancreas and kidney; two tissues which previously did not appear to contain cyto-

