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Cloning And Tissue Distribution Of Subunits C, D, And E Of The Human Vacuolar H^+-ATPase[1]

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The vacuolar proton ATPase (V-ATPase) translocates protons into intracellular organelles or across the plasma membrane of specialised cells such as osteoclast and renal intercalated cells. The catalytic site of the V-ATPase consists of a hexamer of three A subunits and three B subunits which bind and hydrolyse ATP and are regulated by accessory subunits C, D and E. cDNAs encoding subunits C, D, and E were cloned from human osteoclastoma, a tissue highly enriched in osteoclasts, as a first step in the characterisation of the V-ATPase used by the osteoclast. By Northern blot analysis only one mRNA species was detected for each of these subunits, which is consistent the constant transcription level in all tissues irrespective of the presence of specialised cells highly enriched in V-ATPases.

The vacuolar-type of H^+-ATPase (V-ATPase) is responsible for the acidification of endosomes, lysosomes, chromaffin granules and other intracellular organelles in eukaryotic cells [reviewed in 1 and 2]. It consists of a multisubunit complex with cytosolic V_1 and transmembrane V_0 domains [1, 2]. The V_1 domain, containing the ATP catalytic site, is composed of a hexamer of three subunit A (65-77 kDa) and three subunit B (55-60 kDa) proteins plus accessory subunits C, D, and E [1]. The role of accessory subunits C, D and E in V-ATPase function is not fully understood however, a number of studies indicate that subunits C and E may be important in V-ATPase function. Combination of purified subunits A, B, C and E promotes ATPase activity of the V_1 subcomplex in vitro [3] and strong evidence suggests that the V_1 subcomplex is also involved in attachment to the membrane-embedded V_0 domain [4]. Furthermore, the yeast homologs of subunits C and E are essential for the assembly and the activity of the yeast V-ATPase [5].

[1] The nucleotide sequences reported in this paper have been submitted to the GenBank/EMBL Data Bank with accession numbers : X69151, X71490, X71491.

The abbreviations used are: bp, base pair(s); H^+-ATPase, proton translocating adenosine triphosphatase; kb, kilobase(s); kbp, kilobase pair(s); V-ATPase, vacuolar H^+-ATPase.

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Additionally, subunit D may be involved in coupling ATP hydrolysis and proton pumping [3].

The ruffled border of resorbing osteoclasts is known to contain a V-ATPase oriented such that protons are pumped into the extracellular lacuna formed between the resorbing osteoclast and the bone surface, thus facilitating bone resorption by demineralizing the matrix and activating lysosomal enzymes [12, 13, 14]. In an effort to understand the function of the V-ATPase in the ruffled membrane of the osteoclast and to isolate cDNAs which may encode novel isoforms of subunits C, D, and E, we have screened a cDNA library made from human osteoclastoma, a tumor rich in osteoclasts [15, 16].

MATERIALS AND METHODS

mRNA and cDNA preparation

Total RNA was isolated from a sample of osteoclastoma tumour, or the human monocytic cell line THP1 according to an established protocol [17] and poly(A)$^+$ RNA purified by oligo(dT)-cellulose chromatography [18]. Synthesis of single stranded cDNA was performed as described previously [11].

Polymerase Chain Reaction (PCR) amplification of human subunits C, D and E

Oligonucleotide primers 40N (5' - GAC AAG CTT AAA GTG GCT CAA/G TAT/C ATG GC - 3') and 40C (5' - GAC GGA TCC GTT/C GTT/C CTT/C ATG/A ATG GGG AT - 3') were designed, based on bovine brain and a partial human brain cDNAs coding for subunit C [19]; primers 39N (5' - CAG AAG CTT GAG TTC CGT CAT/C ATG CGA/T/G/C AA - 3') and 39C (5' - GGG GGA TCC GTT GCG ACA T/CTC T/CTG T/CTC T/CTT - 3') were based on a cDNA for subunit D isolated from bovine brain [20]; primers 31N (5' - GAC AAG CTT AAG GCA GAA GAA/G GAA/G TTT/C AA - 3') and 31C (5' - GAC GGA TCC GCG TCC CCA/G TTA/G TAA/G/T ATT/C TC - 3') were based on a bovine brain cDNA coding for subunit E [19, 21]. Underlined sequences correspond to restriction sites, *HindIII* and *BamHI*, respectively, to facilitate subcloning [18]. PCR amplifications were performed according to Zwickl [22] with the following cycles: 1) 91^o C /10 sec, 41^o C /30 sec, 72^o C /1 min. for 5 cycles, 2) 91^o C /10 sec, 48^o C /30 sec, 72^o C /1 min for 25 cycles, and 72^o C /10 min with primers 40N and 40C; 1) 91^o C /10 sec, 43^o C /30 sec, 72^o C /1 min. for 5 cycles, 2) 91^o C /10 sec, 50^o C /30 sec, 72^o C /1 min for 25 cycles, and 72^o C /10 min with primers 39N and 39C; 1) 91^o C /10 sec, 39^o C /30 sec, 72^o C /1 min for 5 cycles, 2) 91^o C /10 sec, 46^o C /30 sec, 72^o C /1 min for 25 cycles, and 72^o C/10 min with primers 31N and 31C.

cDNA-library screening, cDNA cloning, and sequence analysis

A cDNA library made from a human osteoclastoma tumour in the phage vector λgt11 was prepared and screened as described [11]. The *EcoRI* inserts of positive clones were excised and subcloned into the plasmid vector pBluescript (KS$^+$; Stratagene)[18]: both strands were fully sequenced using the 70700 Sequenase version 1.0 DNA sequencing kit (USB).

Northern blot analysis

mRNA (2 μg) from human osteoclastoma or human multiple-tissue Northern blots (Clontech) were hybridised under stringent conditions to random prime labelled cDNA probes.

RESULTS AND DISCUSSION

To obtain probes corresponding to the cDNAs coding for subunits C, D, and E of the vacuolar proton pump, we designed degenerate oligonucleotide primers based on bovine cDNA sequences, except for subunit C for which a partial human cDNA sequence was known [19, 20, 21]. Polymerase chain reaction (PCR) amplification of human osteoclastoma cDNA was used to produce probes corresponding to each subunit. The PCR products were subcloned and sequenced to confirm they coded for a human subunit of V-ATPase. These were then used as probes to screen a human osteoclastoma library. Positive clones (one each for subunits Cand D and five for subunit E) were identified, subcloned and sequenced (Figs. 1, 2 and 3). All subunit cDNAs contained Kozak consensus sequences with conserved purines in the -3 position (Figs. 1-3)[23] and in-frame stop codons 5' of the ATG codon (marked with asterisks in Figs. 1 and 2). The predicted molecular weights for each subunit are: subunit C, 41,941 Da; subunit D, 31,704 Da; and subunit E, 26,230 Da.

The open reading frames of subunits C, D and E cDNAs are highly homologous to their bovine counterparts at the nucleotide and amino acid levels having 94% and 99%, 95% and 99%, 92% and 99% homology, respectively, a trend also found between human and bovine subunit A [11] and the kidney and brain isoforms of subunit B [10]. Within the 3' untranslated regions of bovine and

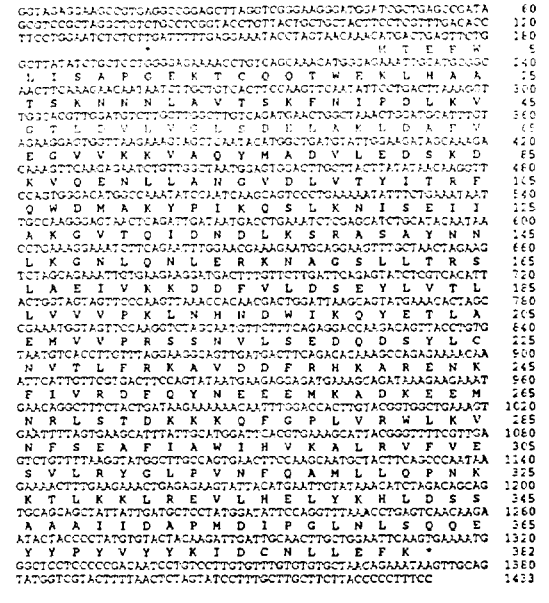

Figure 1. Nucleotide and predicted amino-acid sequence of human subunit C. Asterisks refer to stop codons. This sequence is available in the GenBank/EMBL Data Library with the accession number X69151.

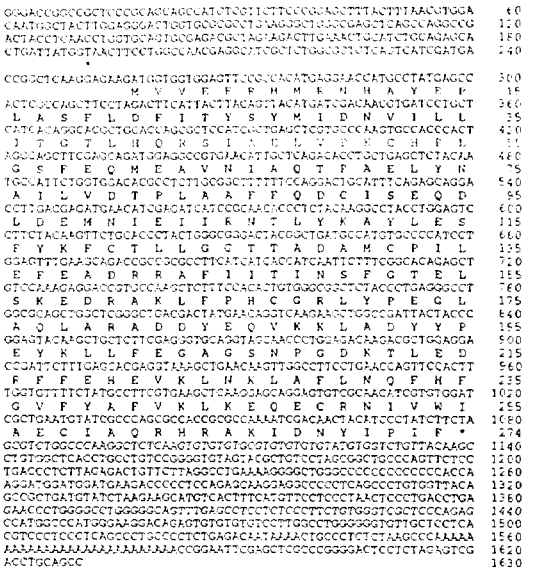

Figure 2. Nucleotide and predicted amino-acid sequence of human subunit D. Asterisks refer to stop codons. This sequence is available in the GenBank/EMBL Data Library with the accession number X71490.

human subunit C, D and E cDNAs, low homologies were found if gaps were inserted to achieve optimal alignment of the sequences. Furthermore, the 3' ends of subunits C and E are identical to partial sequences from human brain [19].

The expression of subunits C, D and E in human tissues was determined by Northern blotting (Fig. 4). The probe for subunit C identified a major band of

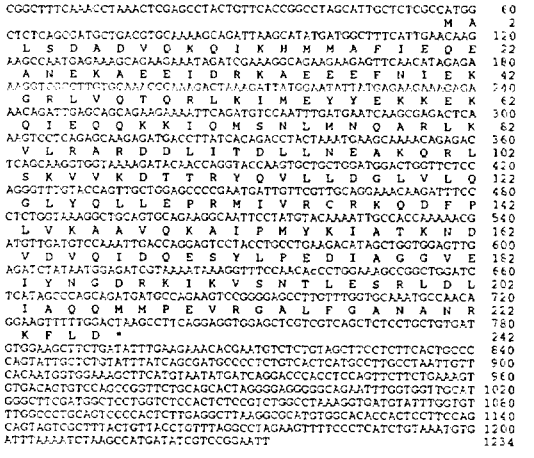

Figure 3. Nucleotide and predicted amino-acid sequence of human subunit E. Asterisk refers to stop codon. This sequence is available in the GenBank/EMBL Data Library with the accession number X71491.

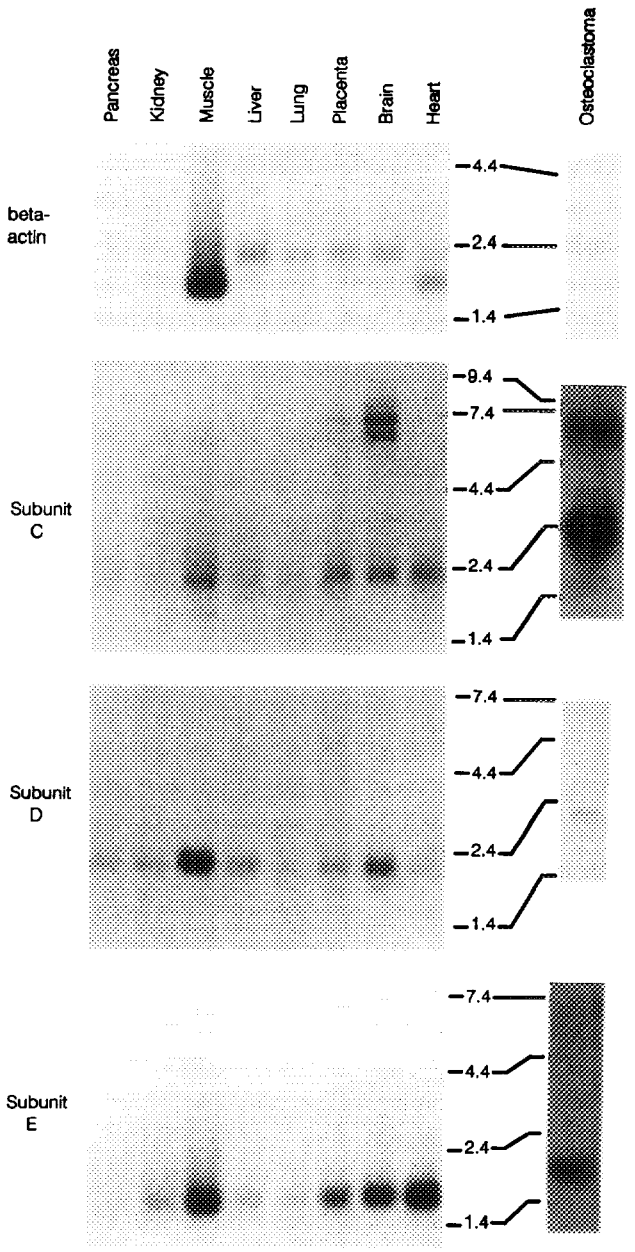

Figure 4. Northern blots of human tissue samples with β-actin and subunit C, D and E probes. Multiple tissue Northern blots (Clontech) and poly(A)$^+$ RNA (2 μg) from human osteoclastoma tumour were hybridised with probes corresponding to β-actin (4 hours exposure), subunit C (18 hours exposure), subunit D (18 hours exposure) and subunit E (5 days exposure). Markers in kb are indicated to the right of the panels.

1.9 kb in all tissues and a doublet of larger mRNAs of 6.0-7.0 kb were clearly present in brain, but also detectable in all tissues upon longer exposure (data not shown). Such a result might suggest the presence of isoforms for subunit C

expressed in a tissue-specific manner. However, the stringent washes of the Northern blots permits hybridisation of the probe only to highly homologous transcripts. Thus, the longer transcripts we observed for subunit C probably represent unspliced nuclear forms of mRNA (due to the method of RNA preparation) or may represent transcripts encoded by the same gene but differing in the length of their 3' untranslated region. Probes for subunits D and E each identified a single band of 1.8 and 1.6 kb, respectively, in all tissues analysed. Low stringency washes of Northern blots for all three subunits did not reveal any other bands. The relative expression of each subunit was measured and compared to the transcript level for a control mRNA, β-actin. In the heteromeric, active V-ATPase the ratio of subunits is 1:1:1 [1]. Subunits C, D and E were expressed at comparable levels in all tissues, irrespective of whether tissues such as brain [2], kidney [6, 8], placenta [24] and osteoclastoma [12, 13, 14] contained cell populations highly enriched in V-ATPases. This suggests a closely related if not identical transcriptional regulation of the corresponding genes, although subunit D appeared to be somewhat less abundant in osteoclastoma, placenta and heart. We cannot exclude that an additional subunit D isoform may exist, although repeated screening of the osteclastoma library did not reveal any other cDNA for this subunit. Furthermore, as the Northern distribution of subunit D mRNA appears to differ slightly from subunits C and E, we are investigating whether subunit D isoforms exist by screening other cDNA libraries. Transcripts for subunits A and B have been shown to be overexpressed in tissues enriched in V-ATPases [11; van Hille et al., manuscript submitted] and if mRNAs for subunits C, D and E follow the same process of amplification, we cannot exclude that additional isoforms of subunits C, D, and E may exist. We have previously used low stringency hybridisation conditions to identify subunits with more than 75% homology at the DNA level [11]. Thus, if isoforms related to C, D and E cDNA sequences exist, they must be much more divergent in sequence and would be very difficult to identify using the same low stringency screening approach that proved successful for the cloning of subunits A and B.

Thus, the cDNAs coding for subunits C, D, and E appear to be ubiquitously expressed in all tissues and in addition, mRNAs for subunits C and E are abundantly expressed in osteoclastoma, a tumour enriched in osteoclasts.

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