Heterogeneity of vacuolar H$^+$-ATPase: differential expression of two human subunit B isoforms

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The catalytic domain of the vacuolar proton ATPase is composed of a hexamer of three A subunits and three B subunits. Here we describe the cloning and characterization of a cDNA isoform of subunit B, HO57, from an osteoclastoma cDNA library. HO57 is represented by three species of mRNA of 1.6, 2.6 and 2.8 kb and is expressed at low levels in a range of human tissues, but at significantly higher levels in brain, kidney and osteoclastoma, and is probably an ubiquitously expressed isoform. In contrast, the kidney-specific isoform has an mRNA of 2 kb and is specifically expressed at high levels only in kidney and, at a lower level, in placenta. Thus the HO57 isoform is integral to the vacuolar ATPase found in the general secretory system of all cells as well as in vacuolar-ATPase-rich sources such as neurones and osteoclasts, whereas both the kidney-specific isoform and HO57 are highly expressed in the kidney. Furthermore, we show by *in situ* hybridization that HO57 is the only isoform that is exclusively and highly expressed by osteoclasts.

INTRODUCTION

The acidification of endosomes, lysosomes, chromaffin granules and other intracellular organelles in eukaryotic cells is mediated by a vacuolar-type H$^+$-ATPase (V-ATPase) (reviewed in [1,2]). V-ATPases consist of a complex of at least nine different subunits distributed between the V_1 cytosolic and V_0 transmembrane domains [1,2]. The V_1 domain is composed of a hexamer of three A subunits (65–77 kDa) and three B subunits (55–60 kDa) which comprise the ATP catalytic site along with accessory subunits of 40, 39 and 33 kDa [1]. The V_0 domain is comprised of a hexamer of 16 kDa subunits plus accessory subunits of 110 and 21 kDa. Not only do V-ATPases serve a general function to acidify vacuoles in a wide variety of cells, but they also appear to cycle between secretory vesicles and the plasma membrane of certain specialized cells where they act as an outwardly directed proton pump transporting hydrogen ions into the extracellular space [3]. Thus V-ATPases have been found to be densely clustered on the plasma membranes of specialized proton-secreting cells such as the renal intercalated cell [4,5], apical cells of the epididymis [6], placental cells [7], osteoclasts [8–11] and the midgut goblet cell of the tobacco hornworm (*Manduca sexta*) larva [12]. Since intracellular organelles and plasma-membrane-located V-ATPases exhibit distinct properties [4,10], one possible mechanism for generating such diversity in function might arise from V-ATPases assembled with isoforms of one or more subunits [13].

Consistent with this idea, two subtypes of subunit A have been cloned from an osteoclastoma tumour, a tissue rich in osteoclasts. The tissue distribution of these subunit A isoforms suggests there are ubiquitous and tissue-specific isoforms [14]. Similarly, several cDNAs coding for isoforms of subunit B have been identified in human brain [15,16] and kidney [17], as well as in bovine brain and kidney [16,18]. Moreover, an anlaysis by two-dimensional gel electrophoresis and functional analysis of purified enzyme from kidney microsomes and apical membrane showed microheterogeneities in subunit B, which are reflected in different biochemical properties [4].

V-ATPase has also been implicated in bone metabolism. The ruffled border of resorbing osteoclasts contains a V-ATPase which pumps protons into the subcellular lacunae, thus facilitating bone resorption [8–11]. To understand the role of V-ATPase in osteoclasts we have undertaken a detailed study of V-ATPase subunits via cloning of subunit cDNAs from human osteoclastoma tumour, which is rich in osteoclast cells [19,20]. Here we describe the cloning and characterization of HO57, a cDNA clone for a subunit B isoform highly expressed in brain, kidney, and osteoclastoma tumour. We find that only two isoforms of subunit B exist and show that HO57 is the only isoform highly expressed in osteoclasts.

EXPERIMENTAL

Starting material, mRNA and cDNA preparation

For RNA extraction a small sample of a human osteoclastoma tumour (weighing approx. 1 g), which was frozen immediately in liquid nitrogen, following surgery, was used. Several small samples of human fetal tissues were also obtained and processed similarly. Total RNA was isolated by the acid/phenol procedure [21] and polyadenylated [poly(A)$^+$] RNA purification by oligo(dT)–cellulose chromatography [22]. Osteoclastoma mRNA (10 μg) in 25 μl of 1 mM EDTA and 10 mM Tris/HCl, pH 8.0, was denatured at 65 °C for 2 min then cooled on ice for 1 min. Synthesis of single-stranded cDNA was performed in a total volume of 50 μl containing the following constituents: 2.5 μg oligo-$dT_{(12-17)}$, 20 units of RNAsin (Promega), 1 mM dNTPs, 10 mM dithiothreitol, 75 mM KCl, 3 mM $MgCl_2$, 50 mM Tris/HCl, pH 8.3, and 800 units of Moloney-murine-leukaemia-virus reverse transcriptase (Gibco–BRL). The reaction mix was incubated for 5 min on ice, 50 min at 37 °C, then divided into 10 μl aliquots and frozen at −20 °C until used.

Abbreviations used: V-ATPase, vacuolar H$^+$-ATPase; H$^+$-ATPase, proton-translocating adenosine triphosphate; p.c., *post coitum*; poly(A)$^+$, polyadenylated.

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PCR amplification of human subunit B

Oligonucleotide primers B1703 [5′-ACT GAA GCT TCA TCT ACC CCG AGG AGA TGA T-3′] and B2593 [5′-CTG ACT CGA GCT CAA ACT TCT GCA GGA ATT C-3′] were based on conserved stretches of nucleotides between the sequence of subunit B isolated from human kidney [17] and brain tissues [15], the fungus *Neurospora crassa* [23] and the flowering plant *Arabidopsis thaliana* [24]. Primer B3002 [5′-CTG ACT CGA GGC ATG AAC CTT CAG TTC TTC-3′] was designed according to the subunit B cDNA obtained from human kidney tissue [17]. Underlined sequences correspond to restriction sites, *Hin*dIII and *Xho*I, used to facilitate subcloning of the amplified products into a plasmid vector.

PCR amplifications were performed as described by Zwickle [25] on 300 ng of cDNA. The amplification reaction was performed on a thermal reactor (Hybaid) with the following PCR cycles: (1) 92 °C/10 s, 51 °C/30 s, 72 °C/60 s for 30 cycles; (2) 72 °C for 10 min. The expected sizes of the amplification products were 890 bp for B1703+B2593, and 1300 bp for B1703-B3002, and they were separated by electrophoresis through a 0.8% agarose/TBE gel [22].

cDNA-library construction, cDNA cloning and sequence analysis

mRNA (10 μg) extracted from a human osteoclastoma tumour was retrotranscribed into cDNA by the method of Gubler and Hoffman [26] using Moloney-murine-leukaemia-virus reverse transcriptase (Gibco). The cDNA was then modified for cloning into the unique *Eco*RI site of the phage vector λgt11, resulting in a cDNA library of 5×10^5 independent phages which was amplified according to standard procedures [22]. Phages (5×10^5) from the human osteoclastoma cDNA library were screened with random-prime-labelled (Boehringer–Mannheim) F890 and F1300 probes. Positive phages were purified by a further three or four rounds of screening. The *Eco*RI inserts of positive λ clones were excised and subcloned into the plasmid vector pBluescript/KS$^+$ (Stratagene). Both strands of three cDNA clones were fully sequenced, using the 70700 Sequenase version 1.0 DNA sequencing kit (USB) [22].

Northern-blot analysis

Samples of mRNA (2 μg) were electrophoresed through a 0.8% agarose/3.7% formaldehyde gel [14] and blotted on to Zetabind membranes (Cuno) according to the protocol of the manufacturer. The Northern-blot membrane was prehybridized in 40% formamide, 5 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 1% SDS, 200 μg/ml salmon sperm DNA, 200 μg/ml tRNA, 10 × Denhardt's reagent for 1 h at 42 °C. Then 1×10^7 c.p.m. of radiolabelled probe (5×10^5 c.p.m./ml of hybridization solution) corresponding to the entire coding region of HO57 cDNA or F1300 fragment was added and hybridization was carried out for at least 18 h at 42 °C. The blots were washed three times for 20 min in 0.2 × SSC and 1% SDS at 65 °C then processed for autoradiography. β-Actin RNA was used as a control for the amount of messenger loaded on to the gel [27].

In situ hybridization

Section preparations

Mouse embryos were isolated from RB (4.15) 4 RMA female mice (Jackson Laboratories) at day 16 p.c. Osteoclastoma tumour from the jaw of a male adolescent was obtained from J. Cournot (Paris). Samples were fixed overnight in a freshly prepared solution of 4% paraformaldehyde/PBS, pH 7.0, at 4 °C and were then placed overnight at 4 °C in 0.5 M sucrose in PBS before storage in liquid nitrogen. Prior to sectioning, tissues were embedded in OCT compound (Miles). Cryostat sections (10 μm thick) were placed on 3-aminopropyltriethoxysilane-treated slides [29] and stored at −70 °C. Prior to hybridization with RNA probes, the sections were dried for 5 min with 0.2 M HCl at room temperature, treated for 30 min with 2 × SSC at 70 °C, dehydrated with increasing concentrations of ethanol (65, 85, 95 and 100%) and finally air-dried. All solutions were treated with 0.1% diethyl pyrocarbonate and autoclaved where appropriate.

Preparation of probes

PCR-generated fragments specific to HO57 cDNA, between nucleotides 514 and 1381, and to K57 cDNA, between nucleotides 519 and 1387 [17], were used to synthethize riboprobes after their cloning into a pBluecript vector. Sense and antisense RNA probes were labelled with [α-^{35}S]UTP (1200 Ci/mmol; New England Nuclear) using T7 or T3 RNA polymerase and according to the supplier's directions (Boehringer–Mannheim).

Hybridization

Prehybridization was performed at 54 °C for 3 h in 50% formamide/10% dextran sulphate/0.3 M NaCl/10 mM Tris/10 mM sodium phosphate (pH 6.8)/20 mM dithiothreitol/0.2 × Denhardt's reagent/0.1 mg/ml *Escherichia coli* RNA and unlabelled 0.2 mM UTP. Hybridization was carried out overnight in the same mixture supplemented with 2×10^5 c.p.m. of [α-^{35}S]UTP-labelled RNA probe in a humidified chamber at 54 °C. Slides were washed twice for 1 h in hybridization solution without dextran sulphate, RNA and unlabelled UTP containing 50% formamide and 10 mM dithiothreitol at 54 °C, then equilibrated for 15 min in a buffer solution consisting of 0.5 M NaCl, 10 mM Tris and 1 mM EDTA, pH 7.5. Sections were then treated with 50 μg of RNAase A in equilibration buffer for 30 min at 37 °C to remove any non-specifically bound probe. Slides were washed in 2 × SSC for 1 h and then in 0.1 × SSC for 1 h. Sections were then sequentially dehydrated for 2 min each in 65, 85 and 95% (v/v) ethanol solutions containing 300 mM ammonium acetate, and 100% ethanol, before being air-dried. Following X-ray autoradiography, the sections were coated with a 1:2 dilution of Ilford K5 photoemulsion, air-dried and exposed for 2 weeks in a light-safe box containing silica gel at 4 °C. Slides were developed in D19 developer (Kodak), rinsed briefly in 2.5% acetic acid, fixed in 30% (w/v) sodium thiosulphate pentahydrate (Fluka), rinsed extensively in water (15 to 30 min) and stained either with Giemsa stain or with haematoxylin/eosin to reveal the cell nuclei.

RESULTS

Cloning of subunit B cDNAs

To obtain a DNA probe corresponding to the transcript of the subunit B gene of the V-ATPase expressed in human osteoclastoma two PCR primers, B1703 and B2593, were designed based on stretches of nucleotide sequences highly conserved between the subunit B cDNAs isolated from human kidney [17] and brain tissues [15], *N. crassa* [23] and *A. thaliana* [24]. Using the PCR technique, any isoform which was predominantly expressed in human osteoclastoma and human fetal brain and bone would be obtained and, in fact, PCR amplification yielded an identical product of 890 bp (called F890) from all three tissues. F890 was cloned, sequenced, and found to show 96%

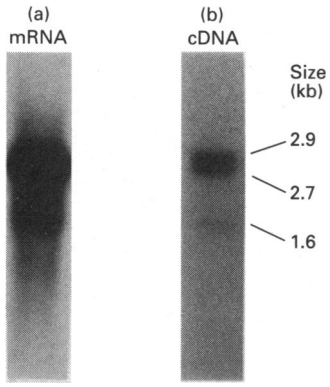

Figure 1 Identification of subunit B transcripts in human osteoclastoma

(**a**) Northern blot of 2 μg of mRNA; (**b**) Southern blot of 500 ng of cDNA hybridized with the probe F890.

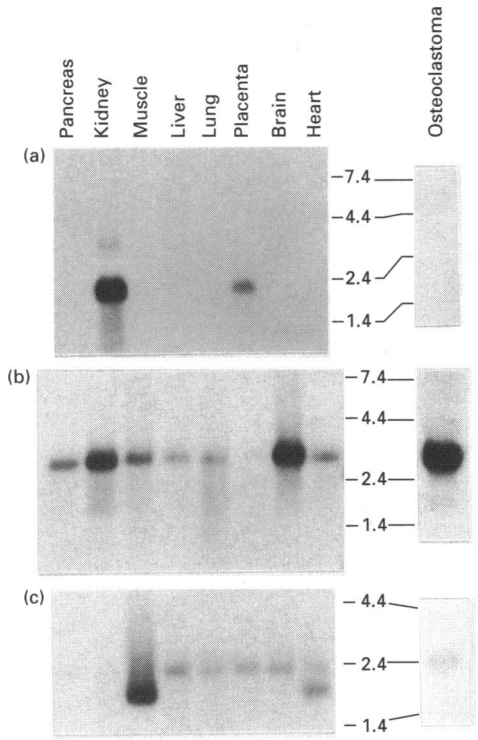

Figure 2 Northern blots of human tissue samples with probes corresponding to subunit B isoforms, F1300 (a), HO57 (b) and β-actin gene (c)

Poly(A)$^+$ RNAs (2 μg) were from a Multiple Tissue Northern Blot from Clontech or isolated from human osteoclastoma tumour. (**a**) Was exposed for 16 h. (**b**) The lane containing osteoclastoma sample was exposed for 4 h, the other lanes for 24 h. (**c**) Was exposed for 4 h. Size markers are indicated.

similarity to the previously published partial human brain subunit B cDNA [15]. On a Northern blot of osteoclastoma mRNA and a Southern blot of cDNA made from human osteoclastoma mRNA, F890 radiolabelled probe detected a doublet of bands at 2.8 and 2.6 kb and a lower band at 1.6 kb which have a similar intensity and size on both blots (Figure 1). When a human osteoclastoma λgT11 cDNA library was screened with the F890 probe, it yielded 18 positive clones. Twelve clones were randomly

Table 1 Relative levels of expression of HO57 and K57 mRNA in human tissues

Abbreviation used: ND, not detectable.

	Tissue								
Ratio	Pancreas	Kidney	Muscle	Liver	Lung	Placenta	Brain	Heart	Osteoclastoma
HO57 / β-Actin	2.9	6.9	0.3	0.4	1.2	0.4	6.1	0.9	39.8
K57 / β-Actin	ND	6.6	ND	ND	ND	0.8	ND	ND	ND

picked and grouped into three classes according to size of insert: 2.8, 2.6 and 1.6 kbp. Sequencing and restriction-enzyme analysis revealed that all clones were closely related, so one clone from each group, namely L2 (2820 bp), L1 (2571 bp) and L5 (1608 bp), was fully sequenced. These three different cDNAs appear to arise from the same gene, since they have identical coding sequences and differ only in the length of the 3′-untranslated region. All three cDNAs started at the same nucleotide in the 5′ untranslated region, which suggests that full size cDNAs had been obtained. The sequence of the longest clone, HO57, has been deposited in EMBL Nucleotide Sequence Database under the accession no. L35249. The sequence of HO57 is 95% homologous to a partial sequence coding for an isoform of subunit B isolated from brain [15], and 76% homologous to a kidney isoform (K57) [17]. This indicates the existence of several subtypes of cDNA coding for subunit B.

In order to study the K57 isoform of subunit B, a fragment of the cDNA coding for the K57 isoform of subunit B was generated by PCR amplification of human fetal kidney cDNA. One oligonucleotide primer, B3002, is specific to the K57 isoform cDNA [17], and B1703 is common to both HO57 and K57 isoform cDNAs. These yielded a PCR fragment of 1300 bp (F1300) that was identical with K57 cDNA.

Tissue distribution of subunit B isoforms

To establish that several isoforms of subunit B exist, the tissue-specific distribution of HO57 and K57 in human osteoclastoma and other tissues was examined by Northern blotting. The probe for the K57 subtype identified a single band of 2.0 kb strongly expressed in kidney, and more weakly in placenta (Figure 2a). By contrast, HO57 identified a major band of approx. 2.6–3 kb in all tissues and a weak band of 1.6 kb (Figure 2b), consistent with the same pattern from osteoclastoma (Figure 1). Interestingly, the bovine mRNAs for brain and kidney subunit B isoforms have identical transcript sizes of 3.0 kb [16]. The level of expression of each subunit-B isoform was then determined relative to that of β-actin control mRNA (Figure 2c) by scanning densitometry and expressed as a ratio (Table 1). This revealed that the HO57 subunit B was expressed at a basal level in all tissues, even those not enriched in V-ATPase and was highly expressed in both kidney and brain, tissues which contain cells with specialized structures enriched in V-ATPases [2,16]. Moreover, HO57 expression in osteoclastoma was at least 6-fold higher than in kidney and brain and probably corresponds to the higher number of osteoclasts found in this tumour (Table 1). Since Northern

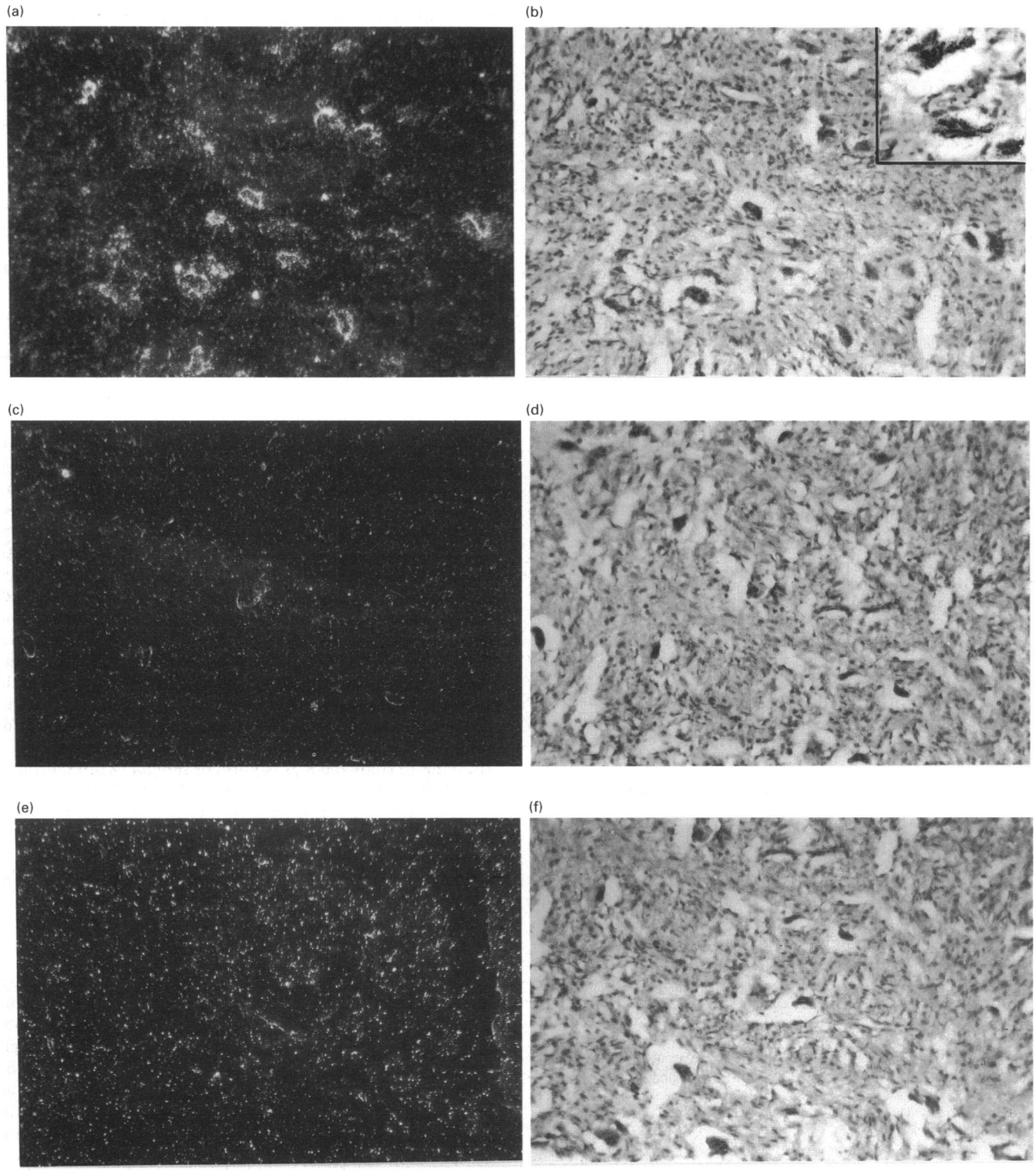

Figure 3 ***In situ*** **hybridization analysis of subunit B expression in human osteoclastoma**

Dark-field (**a**, **c** and **e**) and bright-field (**b**, **d** and **f**) illumination of a section through an osteoclastoma tumour. HO57 antisense riboprobe (**a** and **b**) detected high levels of HO57 transcripts in the multinuclear cells. K57 antisense riboprobe (**c** and **d**) and HO57 sense riboprobe (**c** and **f**) did not detect any specific signal. (**b**) Contains an enlargement of a group of osteoclasts.

blotting does not distinguish between mRNA species of the same size but different sequence, we could not rule out the possibility that HO57 was cross-hybridizing with the brain isoform. To address the expression of isoforms of very similar sequence, i.e. HO57, and the partial brain isoform we designed specific PCR primers for all three cDNA sequences to identify whether all three isoforms of subunit B could be detected. Although the K57 isoform was easily distinguished from the HO57 and partial

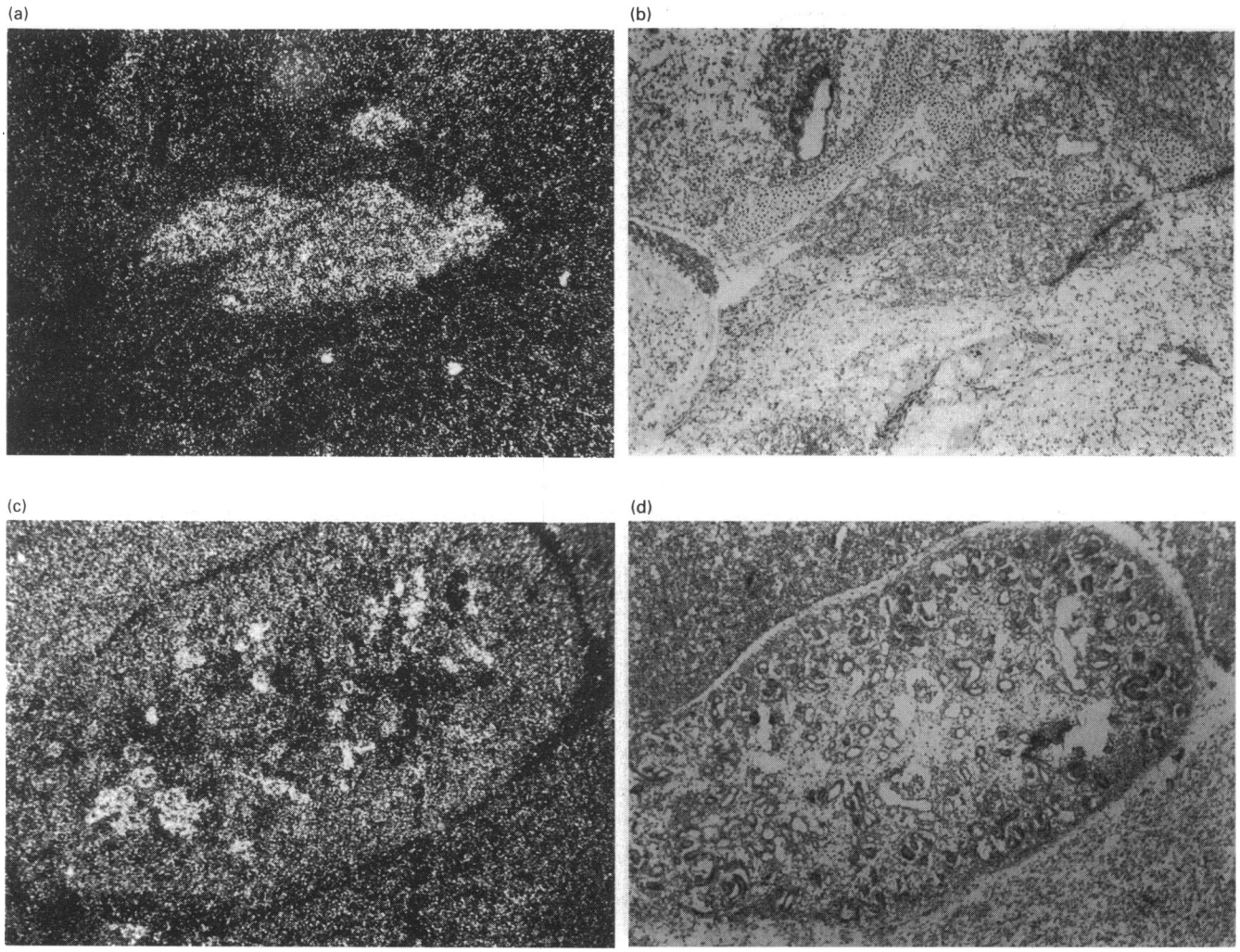

Figure 4 ***In situ*** **hybridization analysis of HO57 expression in the brain and kidney of day-16-p.c. mouse embryo**

Sagittal sections of brain (**a** and **b**) and kidney (**c** and **d**) are shown in dark-field (**a** and **c**) and bright-field (**b** and **d**) illumination.

brain isoforms, it was impossible to obtain PCR products for the partial brain subunit B isoform (results not shown). To address this question we then performed RNase A protection experiments with probes specific for the divergent 5′-end of each isoform. This confirmed that the K57 isoform was expressed in kidney whereas HO57 was expressed in all tissues tested (results not shown). Hence, these experiments corroborated the Northern data and were consistent with a specific isoform in the kidney (K57) and an ubiquitously expressed isoform (HO57) which appears to be the major isotype in osteoclastoma.

As these analyses were underway, the full sequence for the human brain isoform was published [16]. Comparison of this sequence with the human brain partial cDNA and HO57 revealed that the full-length sequence from human brain was identical with that of HO57, except for the 223 bp longer 3′-untranslated region in the HO57 cDNA. The full brain sequence also differed by 5% from the partial brain cDNA [15].

In situ hybridization

The limitation of Northern blots is the inability to determine the distribution of mRNA isoforms within a heterogeneous population of cells such as found in osteoclastoma tumour. To clearly identify whether the osteoclast is the cell type expressing high levels of HO57 in osteoclastoma and also bone tissue, we performed *in situ* hybridization experiments on osteoclastoma and mouse embryo sections. Sense and antisense RNA probes spanning the same region in K57 and HO57 isoforms were used in the hybridizations to human osteoclastoma tumour. The specific antisense probe for the HO57 isoform showed high levels of subunit B transcripts only in the large multinuclear osteoclast cells, while the surrounding mononuclear stromal cells exhibited signals at background levels (Figures 3a and 3b). The antisense probe for K57 (Figures 3c and 3d) and the sense probe for HO57 isoform (Figures 3e and 3f) did not show any specific hybridization. To confirm the Northern data and corroborate the *in situ* analysis on human osteoclastoma, we also examined the expression pattern of HO57 isoforms in mouse embryos at day 16 p.c. (*post coitum*). Day 16 embryos were chosen because, at this stage, bone modelling has begun and would permit the analysis of which subtype is expressed in the active osteoclasts involved in bone modelling, as well as the expression pattern elsewhere, such as in kidney and brain. The human probes used above were utilized for hybridization to mouse embryo sections as

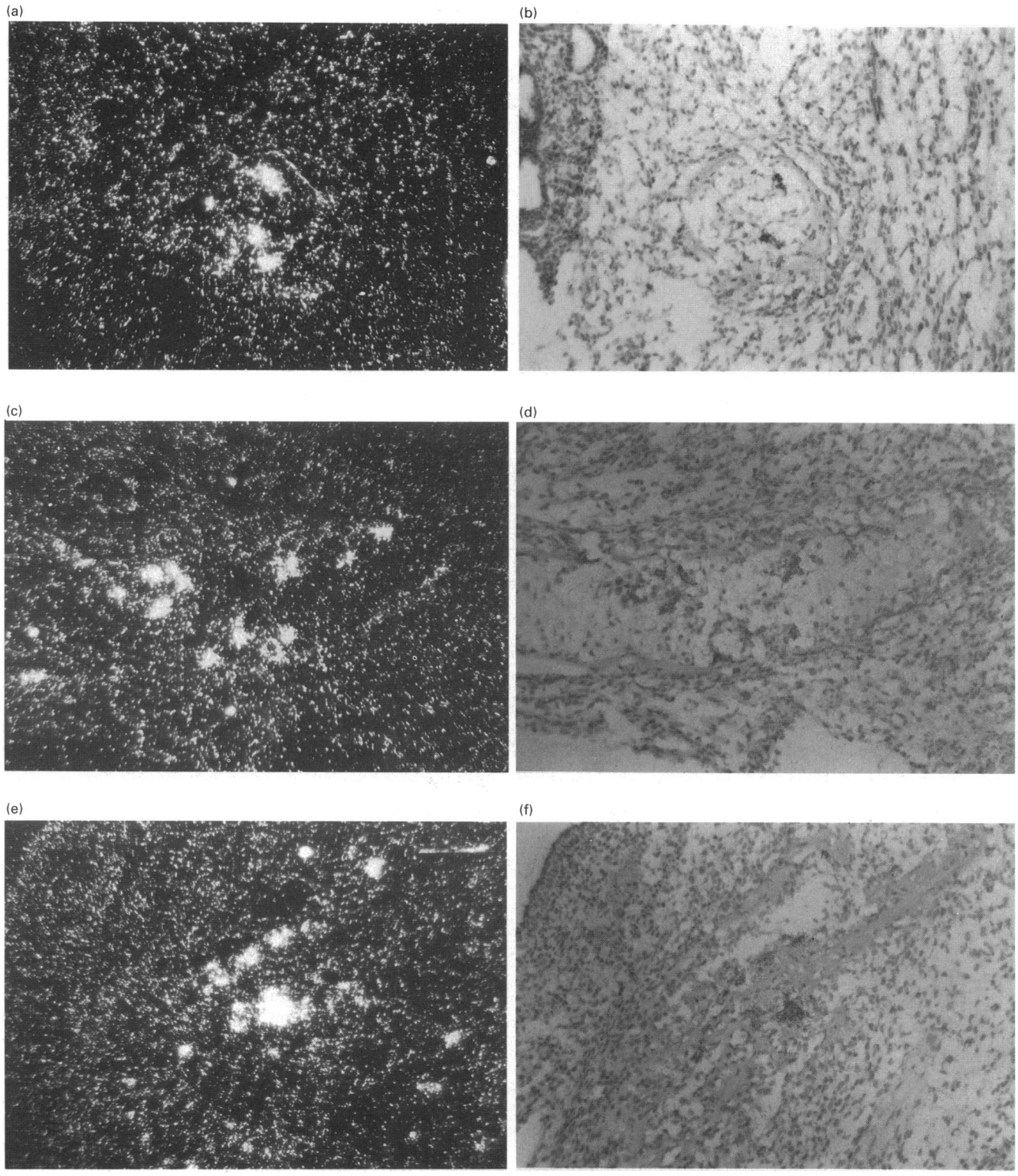

Figure 5 ***In situ*** **hybridization analysis of HO57 expression in vertebrae, rib and lower jaw bone of day-16-p.c. mouse embryo**

Sagittal sections of vertebrae (**a** and **b**), rib (**c** and **d**) and lower jaw bone (**e** and **f**) are shown in dark-field (**a**, **c** and **e**) and in bright-field (**b**, **d** and **f**) illumination.

they recognize mRNAs of the correct size on Northern blots of mouse tissue mRNAs (results not shown). In the day-16-p.c. mouse embryo, high levels of subunit B mRNA were observed in the fifth cranial ganglia of brain (Figures 4a and 4b) and in the developing tubule network of the kidney (Figures 4c and 4d), namely those tissues enriched in V-ATPases. Furthermore, high

levels of HO57 transcripts were detected in the large cells within the vertebrae (Figures 5a and 5b), rib (Figures 5c and 5d) and lower jaw bone (Figures 5e and 5f), with morphological features characteristic of osteoclasts. Only background levels were observed with the sense probes (results not shown).

DISCUSSION

To identify V-ATPase subunit isoforms that are potentially bone-specific, we screened a cDNA library made from osteoclastoma, a tumour rich in osteoclasts. A number of cDNAs coding for the same subunit B isoform were obtained which shared the same coding sequence but differed in the length of the 3′-untranslated region. On the basis of a comparison with previously published sequences for the kidney cDNA isoform and a partial brain cDNA isoform, HO57 was found to show the greatest sequence similarity to the brain isoform, showing microheterogeneities in sequence (95%), while it differed significantly from the K57 isoform (74%). As our aim was to identify and characterize potential osteoclast-specific H^+-ATPase subunits, we performed experiments to determine which isoform of subunit B was expressed in human osteoclastoma and by osteoclasts themselves.

To determine the tissue distribution of each subunit B isoform we used Northern blotting with isoform-specific probes, followed by quantification of the signals by scanning densitometry. Interestingly, the K57 subtype originally thought to be exclusively expressed in kidney tissue [16] was observed at significant levels in placenta. Simon et al. [7] have demonstrated the presence of V-ATPase on the ruffled border of placental vesicles, and our result suggests that the isoform of subunit B expressed in placental cells could be closely related to, if not identical with, the K57 isoform. HO57 was found in all tissues analysed, suggesting that this isoform is ubiquitously expressed, but at much higher levels in kidney, brain and osteoclastoma. Both kidney and brain are known to possess cells with specialized structures that are highly enriched in V-ATPases and therefore have a high level of mRNA, i.e., the apical plasma membrane of renal intercalated cells [16,30] and chromaffin granules in presynaptic cells [2]. Given the structural and functional diversity of kidney, our finding is not surprising. Brown et al. [30] have demonstrated by immunocytochemistry that A and B intercalated cells possess V-ATPase located on apical and basolateral sides of the cell. This probably reflects the dynamic needs of specialized kidney cells for different V-ATPases, thus necessitating different isoforms of subunit B. This issue should be resolved by a detailed analysis of kidney cells using immunocytochemistry and *in situ* hybridization. Osteoclastoma is proposed to be enriched in apparently normal osteoclast cells [19,20], which account for up to 50% of the tumour volume and are known to express high levels of vacuolar ATPase [4]. In the absence of any signal for the K57 subtype, we suggest that the HO57 cDNA isoform accounts for the majority of the subunit B mRNA in human osteoclastoma and is highly expressed in the osteoclast. However, it is clear that any cell requiring V-ATPase for normal cell function [1,2,13], would express subunit B at low levels, which would account for the background levels of the HO57 isoform expressed in all other tissues.

During the course of this analysis, Nelson et al. [16] published the full-length sequence for a brain isoform of subunit B isolated from a human kidney cDNA library. This cDNA clone was identical with HO57. Analysis of the sequence differences between HO57 and the partial brain isoform indicates that the changes in sequence are most likely due to misreading of the sequence. Furthermore, all data obtained by analysis of expression of the kidney and HO57/brain subunit B isoforms are consistent with the existence of only two subunit B cDNA isoforms. These results suggest that the observed changes in gel mobility for subunit B in SDS/PAGE gels [4] are likely to arise from post-transcriptional modification.

The ruffled border of resorbing osteoclasts is believed to contain high levels of V-ATPase which are needed to generate the low extracellular pH required in the bone resorption process [8–11]. Our main focus is to determine the subunit composition of the V-ATPase in osteoclasts. Since the HO57/brain isoform was predominant in human osteoclastoma, it was necessary to identify the cell type expressing high levels of this isoform. To this end we performed *in situ*-hybridization analysis of human osteoclastoma and also day-16-p.c. mouse embryo sections. *In situ* analysis of human osteoclastoma sections confirmed the presence of the HO57/brain isoform of subunit B in the large multinucleate osteoclasts, but not in the mononuclear stromal cells of the tumour. No hybridization signals for the K57 isoform were seen. To confirm the specificity of HO57/brain and K57 probes we then performed *in situ* hybridization on mouse embryos. Strong signals for HO57 were associated with cells in those tissues already shown to contain high levels of this mRNA (Northern blotting), i.e., cranial ganglia V in brain, the developing tubule network in the kidney and large multinuclear cells with clear morphological features of osteoclasts in vertebrae, ribs and lower jaw bone. Thus the high levels of expression of the HO57/brain isoform in human osteoclastoma and mouse embryos correlated strongly with osteoclasts.

It has previously been suggested that the different functions and localization of V-ATPases in cells could be imparted by isoforms expressed in specific organelles or cells. On the basis of our results we propose that the HO57 isoform of subunit B forms part of the V-ATPase responsible for the basal proton-pumping activity present in the general secretory system of all cells; hence the signal is also seen in kidney cells in addition to that of K57. HO57 probably corresponds to the protein isoform observed in the microsomal fraction of renal intercalated cells [4,5,16,30]. Our results also suggest that the V-ATPase with the HO57/brain isoform might be associated with the accumulation of neurotransmitters in the chromaffin granules of brain cells and is probably present on the ruffled border of the osteoclast. By contrast, the alternative K57 isoform of subunit B is probably restricted to the intercalated cells of the kidney and also placental cells. Interestingly, the osteoclast appears to use only the HO57 isoform of subunit B for both its basal intracellular secretory function as well as for acidification of the extracellular lacuna. How the spatial and temporal control of these two divergent processes is achieved remains to be established.

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REFERENCES

1 Forgac, M. (1989) Physiol. Rev. **69**, 765–796
2 Nelson, N. (1991) Trends Pharmacol. Sci. **12**, 71–75
3 Al-Awqati, Q. (1986) Annu. Rev. Cell. Biol. **2**, 179–199
4 Wang, Z.-Q. and Glück, S. (1990) J. Biol. Chem. **265**, 21957–21965
5 Glück, S. (1992) Am. J. Med. Sci. **303**, 134–139
6 Brown, D., Lui, B., Glück, S. and Sabolic, I. (1992) Am. J. Physiol. C913–C916
7 Simon, B. J., Kulanthaivel, P., Burckhardt, G., Ramamoorthy, S., Leitbach, F. H. and Ganapathy, V. (1992) Biochem. J. **287**, 423–430

8 Blair, H. C., Teitelbaum, S. L., Ghiselli, R. and Gluck, S. (1989) Science **253**, 553–560
9 Väänänen, H. K., Karhukorpi, E.-K., Wallmark, B., Roininen, I., Hentunen, T., Tuukkanen, J. and Lakkarkorpi, P. (1990) J. Cell Biol. **111**, 1305–1311
10 Chatterjee, D., Chakraborty, M., Leit, M., Neff, L., Jamsa-Kellokumpu, S., Fuchs, R. and Baron, R. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 6257–6261
11 Wang, Z.-Q., Hemken, P., Menton, D. and Gluck, S. (1992) Am. J. Physiol. F277–F283
12 Gräf, R., Novak, J. S., Harvey, W. R. and Wieczorek, H. (1992) FEBS Lett. **300**, 119–122
13 Anderson, R. G. W. and Orci, L. (1989) J. Cell Biol. **106**, 539–543
14 van Hille, B., Richener, H., Evans, D. B., Green, J. R. and Bilbe, G. (1993) J. Biol. Chem. **268**, 7075–7080
15 Bernasconi, P., Rausch, T., Struve, I., Morgan, L. and Taiz, L. (1990) J. Biol. Chem. **263**, 6012–6015
16 Nelson, R. D., Guo, X. L., Masood, K., Brown, D., Kalbrenner, M. and Gluck, S. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 3541–3545
17 Sühof, T. C., Fried, V. A., Stone, D. K., Johnston, P. A. and Xie, X. S. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 6067–6071
18 Puopolo, K., Kumamoto, C., Adachi, I., Magner, R. and Forgac, M. (1992) J. Biol. Chem. **267**, 3696–3706
19 Chambers, T. J. (1978) J. Pathol. **126**, 125–148
20 Chambers, T. J., Fuller, K., McSherry, P. M. J. and Pringle, J. A. S. (1985) J. Pathol. **145**, 297–305
21 Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
22 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor
23 Bowman, B. J., Allen, R., Wechser, M. A. and Bowman, E. J. (1988) J. Biol. Chem. **263**, 14002–14007
24 Manolson, M. F., Ouellette, B. F. F., Filion, M. and Poole, R. J. (1988) J. Biol. Chem. **263**, 17987–17994
25 Zwickle, M., Zaninetta, D., McMaster, G. K. and Hardman, N. (1990) J. Immunol. Methods **130**, 49–55
26 Gubler, H. and Hoffmann, B. J. (1983) Gene **25**, 263–269
27 Giovanna, P., Jardine, K. and McBurney, M. W. (1991) Mol Cell. Biol. **11**, 4796–4803
28 Melton, D. A., Krieg, P. A., Rebaghiati, M. R., Maniatis, T., Zinn, K. and Green, M. R. (1984) Nucleic Acids Res. **12**, 7035–7056
29 Rentrop, M., Knapp, B., Winter, H. and Schweizer, J. (1986) Histochem. J. **18**, 271–276
30 Brown, D., Sabolic, I. and Gluck, S. (1992) J. Exp. Biol. **172**, 231–244

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