Molecular Cloning, Expression, and Chromosomal Localization of Two Isoforms of the AE3 Anion Exchanger From Human Heart

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Abstract Cl⁻/HCO₃⁻ exchange contributes to regulation of pH₅ and [Cl⁻] in cardiac muscle, with possible effects on excitability and contractility. We have isolated human heart cDNAs, which encode two isoforms of the anion exchanger AE3. These clones share long portions of common sequence but have different 5' ends encoding distinct amino-terminal amino acid sequences. The longer AE3 polypeptide of 1232 amino acids, bAE3, displays nearly 96% amino acid sequence identity to the rat and mouse AE3 “brain isoforms.” The shorter cAE3 polypeptide of 1034 amino acids in length corresponds to the rat AE3 “cardiac isoform.” The unique N-terminal 73 amino acids of the cAE3 sequence are less well conserved between rat and human. Northern blot analysis with isoform-specific probes revealed the presence of both cAE3 and bAE3 mRNAs in human heart tissue. Both AE3 protein isoforms were overexpressed in Chinese hamster ovary cells and detected by immunoblot with antipeptide antibodies. Immunoblot studies of human cardiac membranes detected only cAE3 polypeptides, which were apparently not susceptible to enzymatic deglycosylation. Injection into Xenopus oocytes of cRNAs encoding either cAE3 or bAE3 produced increased ³⁵Cl⁻ uptake into the oocytes, confirming the ability of both AE3 isoforms to transport Cl⁻. The human AE3 gene was localized to chromosome 2. AE3 may provide a new pharmacologic target for antiarrhythmic and cardioprotective drugs. (Circ Res. 1994;75:603-614.)

Key Words • AE3 • anion exchange • Xenopus oocytes • CHOP cells • chromosomal localization

The electroneutral exchange of Cl⁻ for HCO₃⁻ across the plasma membrane of vertebrate cells is a nearly ubiquitous transport mechanism, which contributes in a cell type–specific manner to the regulation of pH₅, volume, and [Cl⁻]. This activity was first described in erythrocytes, where the band-3 anion exchanger, AE1, was the first eukaryotic membrane polypeptide to which an ion transport function was definitively attributed.¹ The band-3–related AE anion exchanger gene family now comprises three members. In addition to AE1, AE2 and AE3 have now been cloned and characterized from a growing number of species.²³ AE3 has been of particular interest for its tissue distribution, which includes excitable tissues such as brain, heart, aorta, and uterus.⁴⁵ The regulation of pH₅ in the heart has been studied most extensively in sheep Purkinje fibers⁶ and in dispersed cardiac myocytes from rats,⁷ guinea pigs,⁸ and chicks.⁹ Sodium-independent Cl⁻/HCO₃⁻ exchange activity has been implicated in cardiac tissues in the defense against alkali loading. Both Na⁺/H⁺ exchange and Na⁺–dependent Cl⁻/HCO₃⁻ exchange have been implicated in defense against acid loading. These systems maintain pH₅ in cardiac muscle at 7.0 to 7.2. The Cl⁻/HCO₃⁻ exchange system appears to be activated only at pH₅ values above resting pH.¹⁰ Cl⁻/HCO₃⁻ exchange activity also has been proposed as one of several transport mechanisms that maintain the cardiac myocyte [Cl⁻] below its low equilibrium value. The contribution of myocyte [Cl⁻], to the reversal potential for Cl⁻ ions, estimated to range between −65 and −45 mV under normal physiological conditions,¹¹ may help determine when during the cardiac action potential the opening of Cl⁻ channels results in membrane hyperpolarization or depolarization. Inhibition by the stilbene inhibitor 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS) of Cl⁻/HCO₃⁻ exchange and of conductive Cl⁻ transport has been found to protect against cardiac arrhythmias associated with ischemia and posts ischemic reperfusion.¹² The molecular identity of the cardiac Cl⁻/HCO₃⁻ exchanger has not been proven, but AE3 has been cloned from rat heart,¹³ and its expression has also been detected in mouse heart.⁴ In view of the importance of Cl⁻ and HCO₃⁻ homeostasis to cardiac function and as part of our continuing investigation of the molecular cell physiology of the AE family of anion exchangers, we have cloned and sequenced two human AE3 isoforms from cardiac cDNA libraries. We have developed specific antipeptide antibodies with which we have detected both recombinant and native AE3 proteins. We have demonstrated that both bAE3 and cAE3 proteins transport Cl⁻. Finally, we have localized the human AE3 gene to chromosome 2.

Materials and Methods

Bacterial Strains

Escherichia coli strains DH5α, XL-1 blue, and MC1061/P3 were maintained as frozen stocks and grown on LB plates in
the absence or (for plasmid transformation) in the presence of 100 μg/mL ampicillin.

**Cell Lines**

Chinese hamster ovary cells expressing polyoma large-T antigen (CHOP cells) were grown to confluence in plastic dishes in α-MEM +10% fetal bovine serum.

**Materials**

mRNA was isolated with the PolyATtract mRNA isolation system (Promega). Oligonucleotides were synthesized with an automated oligonucleotide synthesizer (Millipore). DNA modifying and restriction enzymes were from New England Biolabs unless otherwise noted. [α-32P]dCTP (specific activity, >3000 Ci/mmol) was from Amersham.

**cDNA Libraries**

A commercial AZAP rat heart cDNA library (Stratagene) was used as template for polymerase chain reaction (PCR) amplification of a fragment of rat AE3. A commercial AZAP human heart cDNA library (Stratagene) was used to isolate the first clone of human bAE3. The missing 5' end of bAE3 and the unique sequence region of cAE3 were isolated by PCR from a λgt10 cDNA library made from human left ventricle, a gift of Dr M. Tamkun.

**cDNA Cloning**

32P-labeled cDNA probes were prepared with random priming (Stratagene). Bacteriophage DNA was transferred to nylon membranes by standard procedure. High-stringency hybridization was performed with the random-primed probes in 50% formamide, 5× SSPE, 5× Denhardt’s solution, and 0.5% sodium dodecyl sulfate (SDS) at 42°C. Labeled filters were washed in 0.2× standard saline citrate (SSC) and 0.5% SDS. Isolation of plaque-purified bacteriophage DNA, plasmid subcloning, and plasmid amplification were by standard procedures. Selected clones were analyzed as below.

**cDNA Sequencing**

Plasmid-sequencing templates were prepared with nested deletions using the Exon II sequencing kit (Stratagene). Sequencing reactions were carried out using the Taq DyeDeoxy Terminator or DyePrimer cycle sequencing kit (Applied Biosystems). The reaction products were sequenced on an automated DNA Sequenator (Applied Biosystems 370A).

**Sequence Analysis**

All sequence analysis was performed using the Genetic Computer Group sequence analysis software (GCG). Sequence data were compiled using the program GELASSEMBLE. Database searches were performed using the FASTA or BLAST programs. Multiple-sequence alignments were performed using the program PILEUP.

**Polymerase Chain Reaction**

Routine PCR amplification for cloning and construction was performed for 35 cycles in a Perkin-Elmer 4800 DNA thermal cycler, using a cycle of 1-minute denaturation at 95°C, 2-minute annealing at 55°C, and 3-minute extension at 72°C. The 100-μL reaction mixture contained template, 20 pmol of each primer, 500 μmol/L dNTP, 2 to 5 U of Taq DNA polymerase (Promega), and the manufacturer’s buffer system.

**Molecular Cloning of Human Cardiac AE3 cDNAs**

A 1.44-kb PCR fragment was amplified from a rat heart library using two 20-mer oligonucleotide primers flanking the sequence 968-2408 of rat AE3. This cDNA was restricted at the Nhe I 1276 and HindIII 2348 sites to generate a 1072-bp cDNA, which was subcloned, amplified, and used as a 32P-labeled cDNA to probe a commercial human cardiac AZAP cDNA library (Invitrogen). Twelve identical clones were isolated (Fig 1a, AzhbAE3, clone 1). Sequence analysis revealed a single large open reading frame incomplete at the 5' end, with very high homology to published AE3 sequences from rat and mouse. The missing 5' end was obtained by PCR from a λgt10 human cardiac library by use of a 3' primer extended from nucleotides 112 to 95 and a 5' λgt10 flanking primer. The amplified fragment was 220 nt in length (not shown) and included the putative initiation codon preceded by a strong Kozak consensus initiation sequence. This fragment contained no restriction site unique for the entire AE3 sequence. Therefore, a longer fragment of 841 nt was amplified from the same library using the 5' λgt10 flanking primer and a 3' primer encoding nucleotides 830 to 813 (Fig 1a, pAgbAE3, clone 2). Direct DNA sequencing of two independent PCR amplification products yielded identical DNA sequence. To reconstruct the full-length bAE3 coding region, this PCR...
Deglycosylation

Postnuclear membranes were extracted in 1% SDS. The supernatant was diluted in 10 vol of 1% Nonidet P-40 and then incubated in the presence or absence of peptide-N-glycosidase F (PNGase F, New England Biolabs) for 1 hour at 37°C as directed by the manufacturer. Alternatively (as shown in Fig 7B and 7C), postnuclear membranes were incubated in the presence or absence of PNGase F in 0.25 mmol/L sucrose, 10 mmol/L Tris-Cl, and 1 mmol/L EDTA, pH 7.5.

Immunoblots

SDS–polyacrylamide gel electrophoresis (PAGE) and electrophoretic transfer to nitrocellulose were performed as previously described.18,19 Blots were incubated with affinity-purified antibodies diluted to a final concentration between 0.08 and 0.12 μg/mL in the presence or absence of 2 μg/mL competitor peptides, followed by incubation with secondary antibody coupled to horseradish peroxidase (Jackson Immunochemicals) and development by enhanced chemiluminescence according to manufacturer’s instructions (Amersham). Protein was measured by the bis-cinchonic acid assay (Pierce).

36Cl– Uptake Measurement Into Xenopus Oocytes

Capped cRNA was transcribed with T7 RNA polymerase (Ambion) from linearized pBluescript plasmids (Stratagene) into which were ligated the cDNAs encoding the complete protein sequences of hbAE3 and hcAE3 (Fig 1). cRNA or water was microinjected into defolliculated Xenopus oocytes, which were then incubated in ND-96.20 Two or 3 days later, oocytes were subjected to assays of 36Cl– influx in medium containing final concentrations of (mmol/L) NaCl 131, KCl 2, CaCl2, 1, and HEPES-Cl 5, pH 7.4. Influx was measured over 60 minutes under conditions earlier found to be linear for recombinant AE1 and AE2 in Xenopus oocytes.20,21 All radiolabeled compounds were from New England Nuclear.

Chromosome Mapping

Genomic DNAs from human IMR-91 cells, mouse 3T6 cells, and Chinese hamster RJK86 cells and from the 25 interspecific cell lines of the National Institute of General Medical Sciences (NIGMS) human/rodent somatic cell hybrid mapping panel 2 (Coriell Institute) were screened by PCR for the presence of the human AE3 gene. PCR primers specific for human AE3 were selected from the putative Cl exon of human AE3 (Fig 2B). The 5’ primer (5’-AGAGCCGAGGGTCTGTTAG-3’) extended from human nucleotides –53 to –34, a region not present in the rat Cl 5 untranslated region (Fig 4A).13 The 3’ primer (5’-CAAAGCCCCAGCTATTGGTA-3’) extended from human nucleotides 82 to 63, a segment that in rat shows only 50% nucleotide identity. PCR was performed in a Perkin-Elmer 9600 thermal cycler, using 40 cycles of 10-second denaturation at 94°C, 30-second annealing at 56°C, and 30-second extension at 72°C. The 8.35-μL reaction volume contained 100 ng genomic DNA, 10 pmol of each primer, 6 μmol/L each of dCTP, dGTP, and dTTP, 1.2 μmol/L of dATP, 0.25 U of Taq DNA polymerase (Promega) in a buffer containing (mmol/L) Tris-Cl 15, pH 8.3, KCl 75, and MgCl2 2.25. Three microliters of each PCR reaction was electrophoresed on a denaturing polyacrylamide gel and subjected to autoradiographic analysis. PCR fragment lengths were determined by direct comparison with a sequencing ladder developed using M13mp18 as a template.

The predicted 135-nt length of the PCR product of human AE3 was confirmed. The identity of this PCR product was further confirmed by nested serial PCR. Use of the two internal primers 5’-GGAGCCGAGGGCCAGTGC-3’ and 5’-GGTGATCCCCAGGGCCAGG-3’ yielded the predicted AE3 PCR product of 99 nt in length.

Detection of RNA

Total RNA was isolated from postmortem human left ventricle in congestive failure (kindly provided by Dr. S. Izumo) by solubilization in guanidinium isothiocyanate followed by cesium chloride gradient centrifugation. Poly(A)1 RNA was isolated from total RNA by the PolyA-Tract mRNA isolation system (Promega) and blotted to nylon membranes as previously described.16 32P-labeled probes were prepared by random oligo priming. High-stringency hybridization was performed at 50°C in the hybridization solution described above, with subsequent washing at 50°C in 0.2× SSC and 0.5% SDS. RNA size standards were from Gibco-BRL.

Antibodies

Three peptides encoding murine bAE3 residues 1216-1227 and 849-862 and human cAE3 residues 42-53 (Fig 2B) were prepared with N-terminal cysteines on an Applied Biosystems synthesizer. Purity was confirmed by high-performance liquid chromatography and amino acid analysis. The cysteine peptides were coupled to keyhole limpet hemocyanin as previously described14 and dialyzed, and the resultant mixtures of coupled and uncoupled peptides were mixed with Freund’s complete adjuvant and injected subcutaneously into New Zealand White rabbits. Boosts were performed with Freund’s incomplete adjuvant. Antisera were affinity-purified by acetic acid elution from a peptide–bovine serum albumin affinity column constructed as previously described.18

Transient Transfection and Cardiac Membrane Preparation

Complementary DNAs encoding each of the two full-length AE3 polypeptides were separately ligated into the eukaryotic expression vector pcDNA1 (Invitrogen). CHOP cells were transiently transfected with these AE3 plasmids as previously described.14 Two days after transfection, CHOP cells were harvested from the Petri dish, washed in 140 mmol/L NaCl and 10 mmol/L sodium phosphate at pH 7.4, solubilized directly in SDS load buffer,19 and frozen for later analysis. Fragments (0.5 g) of the same human heart from which mRNA was isolated as described above were homogenized in 0.25 mol/L sucrose and 10 mmol/L Tris-Cl, pH 7.5, containing 1 mmol/L EDTA, 200 μg/mL phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 1 μg/mL aprotinin, and 1 μg/mL chymostatin. Postnuclear membranes were prepared, directly solubilized in SDS load buffer, electrophoresed, and transferred without freezing.
**cDNA Clone Characteristics**

The composite sequence of human bAE3 is 3933 nt in length, including 11 nt of the 5' untranslated region, 237 nt of the 3' untranslated region, and a continuous open reading frame of 3696 nt (Fig 2A). The sequence lacks a poly(A) tail but extends close to the ends of the published rat and mouse sequences. Although lacking a consensus AATAAA polyadenylation signal, the clone has a candidate signal in the same site as those of the rat and mouse sequences (human nucleotides 3916-3954).

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**Results**

**cDNA Clone Characteristics**

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to 3922). The human 3' untranslated region contains two insertions of 42 and 34 nt not present in the rat (Fig 4B). The former insertion is present in the mouse. If these two insertions are excluded from the comparison, the human and rat 3' untranslated regions show 78% nucleotide sequence identity over the untranslated region from nucleotides 3714 to 3923. This degree of identity suggests possible functional importance of the short 3' untranslated region.

The deduced bAE3 protein sequence predicts a length of 1232 amino acids and an apoprotein molecular weight of 135 705 (Fig 2A). The human bAE3 amino acid sequence is 95% identical to those of rat and mouse AE3 (Fig 3B). The main differences are insertions into the human sequence of a Gly residue at position 245 and of the four residues GSLA in ecto-loop 5-6 (Z-loop). In contrast, and as true for rat and mouse, the conservation among the human AE1, AE2, and bAE3 isoforms is much lower (Fig 3C).

The human bAE3 sequence portrayed by hydropathy analysis has three principal domains. The first is an amino-terminal hydrophilic domain of \( \approx 700 \) amino acids, predicted by analogy with AE1 to be cytoplasmic.\(^3\) The amino-terminal 250 residues of this domain have no corresponding region in human AE1 but share 33% identity (55% similarity) with the proline- and histidine-rich amino-terminal sequence of human AE2. The region encompassing residues 251 and 678 shows 40% identity (62% similarity) with the amino-terminal domain of human AE1 but a much higher (59%) identity (72% similarity) with human AE2 (Fig 3C).\(^2\) The second major domain of human AE3 is the hydrophobic domain of \( \approx 490 \) amino acids, modeled by analogy with AE1 to be a polytopic membrane-spanning region containing up to 14 transmembrane spans. The third major domain of human AE3 is a short hydrophilic C-terminal domain of \( \approx 40 \) amino acid residues, which by analogy with AE1 is expected to be cytoplasmic.

Several features of the human bAE3 sequence deserve additional note. Both lysine residues, which in AE1 can serve as covalent binding sites for the transport antagonist 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS),\(^2\) are conserved in human AE3 (asterisks in Fig 2A) as aa 842-845. Of the two NXS sites of potential N-linked glycosylation, only the second (aa 873-875) is modeled to reside on the ectoplastic face of the protein within the Z-loop.\(^2\) In addition, in the first site (NPS, aa 440-442), the presence of proline between Asn and Ser/Thr would inhibit N-glycosylation.\(^23,24\) In contrast, AE2 contains three putative externally disposed glycosylation sites, also in the Z-loop. AE1 contains only one glycosylation site but in ecto-loop 7-8.

Several consensus sites for phosphorylation by casein kinase II (CKII), protein kinase A (PKA), protein kinase C (PKC), and protein tyrosine kinases are present in the sequence. Putative CKII and PKC sites are numerous. A PKA site at residue 289 is also present in AE2. Possible tyrosine phosphorylation sites at residues 589 and 1146 are also shared with AE2 and AE1. An ATP/GTP binding site motif A (P-loop) is predicted at position 495-502 of the cytoplasmic domain of AE3. This site is present neither in AE2 nor in AE1. Cys 843 of AE1, the site of AE1 palmitoylation, is conserved in AE3 (as Cys 1165) and in AE2. The second heptad of the consensus 4-heptad leucine zipper in the cytoplasmic domain of AE2 has an isoleucine substitution in the AE3 sequence (bAE3 I378). The same residue is replaced by phenylalanine in AE1. Isoleucine/leucine zippers have recently been proposed to mediate hydrophobic helix-helix interactions similar to those of leucine zippers.\(^25,26\) Human AE3 also contains a proline-rich sequence at residues 154-161 that fits the consensus of an SH3 domain binding site.\(^27\) Other portions of the proline-rich cytoplasmic domain might constitute binding sites for additional proteins that contain SH3 domains.

The cAE3 deduced protein sequence, 1034 amino acids in length with an apoprotein molecular weight of 114 249, was a composite of the cAE3 PCR product and the bAE3 sequence with which it shared 478 nt of overlapping DNA sequence. In the rat, both transcripts are products of a single gene.\(^13\) The degree of sequence conservation between the 73 amino acids of the human and rat C1 exons is only 63% (Fig 3A), lower than in any other region of AE3. The 109 bases of the 5' untranslated region are 52% identical to the corresponding region of the rat gene (Fig 4A).\(^13\) If the single 18 nt insertion in the human 5' untranslated region at nucleotides 57 to 75 is excluded from this comparison, identity rises to 62%. The human C1 exon amino acid sequence contains two PKC recognition motifs, SPR and SIR, at positions 34 and 59, respectively (Fig 3A). Neither is present in the rat cAE3 sequence. A single putative CKII phosphorylation site is present in both species.

Expression of mRNA

A Northern blot of poly(A)+ RNA isolated from human heart tissue was probed with three different probes (Fig 5): full-length AE3, an 880-bp 5' probe unique to bAE3, and a 210-bp 5' probe unique to cAE3. The full-length probe hybridized with two transcripts of \( \approx 4.3 \) and 3.7 kb in length (Fig 5b). The 5' bAE3-specific...
Fig 3. A, Amino acid comparison of human and rat13 C1 exon. The upper sequence is the human C1 exon, and the bottom sequence is the rat C1 exon. The percentage of similarity is 81%, the percentage of identity, 63%. B, Alignment of deduced amino acid sequences of mouse4, rat5, and human bAE3. Amino acid residues are numbered at the right. C, Alignment of deduced amino acid sequences of human AE1, AE222, and AE3.
probe hybridized only with the longer transcript (Fig 5a), whereas the cAE3-specific probe hybridized only with the shorter transcript (Fig 5c). Therefore, both bAE3 and cAE3 transcripts are expressed in the human adult heart in congestive heart failure. Northern blots of adult heart from rabbits and rats also revealed both bAE3 and cAE3 transcripts (not shown), whereas in mouse heart only cAE3 mRNA was detectable, as previously reported.4

Immunoblotting of Recombinant Human bAE3 and cAE3 Isoforms

CHOP cells were transiently transfected with bAE3 or cAE3 in the eukaryotic expression vector pcDNA1,
then scraped, and solubilized directly in SDS load buffer. Frozen samples were thawed, electrophoresed, and transferred to nitrocellulose. Two antibodies to AE3 were used as immunologic probes to detect recombinant AE3 proteins. One was raised against the 12 C-terminal amino acid residues of murine AE3, identical to residues 1221-1232 in human AE3. Another antibody was raised against murine AE3 residues 849-862, located in the Z-loop between the conserved putative DIDS-binding lysines\textsuperscript{21} and the putative N-linked glycosylation site. These residues are identical in 13 of 14 positions to human AE3 residues 850-863.

The Z-loop antibody was not a useful immunoblot reagent. In contrast, the C-terminal antibody detected two polypeptides of M\textsubscript{i}, 145 and 165 kD in CHOP cells transfected with bAE3 but not in mock-transfected cells. Recognition was specifically blocked only by the peptide antigen but not by nonspecific peptide (Fig 6A). The upper band (40% of total AE3) may correspond to the mature glycosylated protein located beyond the cis-Golgi and at the cell surface, whereas the lower band (60% of total) may correspond to the immature glycosylated protein in the endoplasmic reticulum. Alternatively, the lower band is a proteolytic degradation product that preserves the C-terminal epitope. The open arrows indicate putative dimeric forms of AE3.

The anti-AE3 C-terminal antibody detected in CHOP cells transfected with cAE3 two or more smaller polypeptides of M\textsubscript{i}, 100 to 140 kD (Fig 6B). The recombinant cAE3 polypeptides were less abundant, more diffusely migrating, and apparently processed less completely to their mature glycosylation states than was bAE3 in CHOP cells. The cAE3 bands may represent two cAE3 glycosylation forms as described above for bAE3, along with intracellular proteolytic degradation product(s) of each. Immunostaining of all four polypeptides was blocked by relevant peptide antigen but not by irrelevant peptide. Dimerization of both AE3 isoforms was promoted by nonionic detergent extraction, by freeze-thaw in any detergent, and especially by boiling in SDS (not shown).

The C-terminal dodecapeptides of AE2 and AE3 are identical in 10 of 12 amino acids. Therefore, before use of the anti-AE3 C-terminal antibody to detect endogenous cardiac AE3 polypeptides, we assessed its isoformal specificity. The AE3 antibody did not detect rat choroid
plexus AE2 (or entrapped red blood cell AE1), whereas only anti-AE3 antibody and not anti-AE2 antibody detected bAE3 in transiently transfected CHOP cells. Both signals were specifically blocked only by peptide antigen and not by irrelevant peptide (not shown). Thus, anti-AE3 C-terminal antibody did not recognize AE2 polypeptide by immunoblot.

**Immunoblot Detection of AE3 in Human Heart Membranes**

Since the endogenous cAE3 polypeptide had not yet been detected and since anti-Z-loop antiserum was not useful as an immunoblot reagent, a second rabbit anti-cAE3 serum was raised against the cAE3-specific peptide 42-53. Fig 7A shows that both this antibody and the C-terminal antibody detected a cAE3 band in human heart postnuclear membranes with appropriate immunspecificity (arrow). At shorter exposure time, it was evident that the cAE3 band of ~115 kD comprised a doublet (not shown). The mobility of the AE3 bands and their reactivity with the cAE3-specific antibody identified them as cAE3 polypeptides. Use of freshly prepared membranes, even from frozen tissue, minimized apparent dimerization of cAE3. The C-terminal antibody did not detect in cardiac membranes bAE3 at the monoclonal size of ~160 kD, observed in transfected CHOP cells (Fig 6A). The several bands that did not show immunspecificity were detected by secondary antibody alone (Fig 7C). In addition, trichloroacetic acid precipitation of cardiac membranes before SDS-PAGE eliminated (or aggregated) these nonspecific bands without altering mobility or recovery of cAE3 on immunoblot (not shown).

Since the deduced amino acid sequence of cAE3 encodes one consensus N-linked glycosylation site with predicted exoplasmic disposition and since both AE1 and AE2 are glycoproteins, enzymatic deglycosylation of human cardiac AE3 was tested. The substrate preparation procedure recommended by the supplier of recombinant PNGase (SDS solubilization and denaturation, followed by dilution of the SDS with 10 vol of 1% Nonidet P-40) led to no reduction of Mobility after a 1-hour deglycosylation reaction (not shown). Since deglycosylation of native AE2 in gastric mucosal membranes is rapid at low ionic strength but inhibited by physiological ionic strength (A. Zolotarev and S.L. Alper, manuscript in preparation), deglycosylation of native cardiac membranes was carried out at low ionic strength. Fig 7B shows that many of the nonspecifically stained bands exhibited reduced Mobility after incubation with PNGase F, whereas cAE3 showed no evidence of deglycosylation, whether detected by anti-cAE3 42-53 or by anti-C-terminal antibody.

**Functional Expression of the Two AE3 Isoforms**

Murine bAE3 has been shown to mediate Cl––HCO3− exchange when transiently expressed in 293 cells. We injected cRNA encoding human bAE3 into *Xenopus* oocytes and measured their ability to increase 36Cl− uptake by the oocytes. Fig 8, top, shows that expression of human bAE3 in oocytes led to increments in unidirectional Cl− uptake. These data document the ability of human BAE3 to transport Cl− and are consistent with its reported function as a Cl−–HCO3− exchanger.

Functional testing of cAE3 has not yet been reported. Therefore, we injected cRNA encoding human cAE3 into *Xenopus* oocytes (Fig 8, bottom). As was

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**Fig 6.** Immunoblot detection of human bAE3 (A) and cAE3 (B) polypeptides in transiently transfected CHOP cells with antibody against AE3 C-terminus. Cells were mock-transfected (lanes 1) or transfected with bAE3 cDNA (panel A, lanes 2 through 4) or with cAE3 cDNA (panel B, lanes 2 through 4). The nitrocellulose strips of both panels were incubated with antibody alone (lanes 1 and 2) in the presence of excess specific peptide antigen (lanes 3) or of excess irrelevant peptide (lanes 4). In panel A, two major bands of 145 and 165 kD (filled arrows) indicate heterologous human bAE3 polypeptides. Putative bAE3 dimers are indicated by open arrows. In panel B, multiple bands extending from 130 to 100 kD (bracket) correspond to heterologous cAE3 polypeptides. The flanking arrowheads mark protein size standards.

**Fig 7.** Immunoblot detection of AE3 in human cardiac membranes. A, Each lane was loaded with 100 μg human cardiac postnuclear membranes prepared from frozen postmortem tissue. Blot strips were incubated with affinity-purified antipeptide antibodies to cAE3 aa 42-53 (lanes 1 and 2) or to the common C-terminal peptide (lanes 3 and 4) in the presence of irrelevant peptide (lanes 1 and 3) or of specific peptide antigen (lanes 2 and 4). The cAE3 polypeptide doublet specifically detected by both antibodies is indicated by the arrow. Other bands present are nonspecific, as defined by reactivity in the presence of specific peptide antigen (lanes 2 and 4) and by reactivity with secondary antibody alone (see panel C). Size standards are indicated in kilodaltons. B, Attempted enzymatic deglycosylation of human cAE3 is shown. Human cardiac postnuclear membranes (100 μg) were treated with (lanes 1 and 3) or without (lanes 2 and 4) peptide-N-glycosidase F in the presence of irrelevant peptide (lanes 1 and 2) or specific peptide antigen (lanes 3 and 4) as described in "Materials and Methods" and then immunoblotted with antipeptide antibodies to cAE3 aa 42-53. C, Bands detected by secondary antibody alone, after omission of primary antibody, are shown.
true for bAE3, cAE3 also conferred increased unidirectional $^{36}$Cl$^{-}$ uptake onto the oocytes. More extensive oocyte experiments with recombinant AE2$^{20}$ have shown that the corresponding incremental Cl$^{-}$ uptake represents Cl$^{-}$-anion exchange.

Chromosomal Localization of the Human AE3 Gene

The murine AE3 gene has been mapped to chromosome 1, ∼35 cM from the centromere, and within 1 cM of the Lsh/Bcg locus. This region of murine chromosome 1 is syntenic with several loci from region q34-37 of human chromosome 2 (White et al, unpublished data, 1993). Therefore, we tested the hypothesis that the human AE3 gene also resides on chromosome 2 by use of NIGMS human/rodent somatic cell hybrid mapping panel 2. A sequence-tagged site corresponding to the human AE3 putative C1 exon was used to type this somatic cell hybrid mapping panel. The 135-nt PCR product corresponding to the human AE3 gene was amplified only from the hybrid line GM/NA10826B, which, alone among the cell lines of the panel, contains human chromosome 2 (Fig 9). The identity of this PCR product as human AE3 was confirmed by nested PCR, which yielded the predicted 99-nt fragment (not shown). This observation is consistent with the localization of the AE3 gene to human chromosome 2. In rat, both bAE3 and cAE3 have been definitively shown to derive from a single AE3 gene.\(^\text{13}\)

Discussion

The present article has presented the molecular cloning, sequencing, detection, and heterologous functional expression of two isoforms of the human AE3 anion exchanger gene, bAE3 and cAE3. The nucleotide and deduced amino acid sequences were highly similar to those previously reported from mouse and rat. The substantial differences between the human and rat cAE3-specific sequences of the C1 exon are reminiscent of the considerable sequence differences between the amino termini of erythroid AE1 proteins from different species.\(^\text{2,3,22}\) Both bAE3 and cAE3 mRNAs were expressed in failing human ventricle. Rabbit and rat heart were also found to express both bAE3 and cAE3 mRNAs, in contrast to mouse heart, which expressed only cAE3 at the level of resolution of Northern analysis.

The human bAE3 and cAE3 cDNAs likely are products of the same gene, for the following reasons. First, the 478 nucleotides 3' to the unique sequence corresponding to the rat C1 exon\(^\text{12}\) of the human cAE3 cDNA were sequenced and found to be identical to the corresponding region of human bAE3. This region spans four exons in the rat gene.\(^\text{13}\) Second, the highly homologous rat bAE3 and cAE3 transcripts arise from the same gene, with the unique C1 exon sequence located within intron 6 of the rat AE3 gene.\(^\text{13}\) Third, the location of the human AE3 gene on chromosome 2, as detected in the present study using a cAE3-specific sequence-tagged site, corresponds to the syntenic region of mouse chromosome 1, to which the single mouse AE3 gene has been mapped by linkage with a bAE3 probe (White et al, unpublished data, 1993). Final proof that bAE3 and cAE3 transcripts issue from a single human gene awaits finding the unique N-terminal cAE3 sequence within an AE3 genomic clone that encodes bAE3.

We developed two antipeptide antibodies to AE3 useful as immunoblotting reagents. One was directed against the C-terminal AE3 peptide common to bAE3 and cAE3 and was used to detect recombinant bAE3 and cAE3 in transiently transfected CHOP cells. This antibody was specific for AE3 and did not detect AE2 or AE1. The second antibody was raised against cAE3 aa 42-53, a sequence not present in bAE3. Both antibodies detected cAE3 in failing human heart membranes by

Fig 8. Bar graphs showing $^{36}$Cl$^{-}$ influx into Xenopus oocytes previously injected with water or with cRNA encoding bAE3 (top) or cAE3 (bottom). Each bar represents 8 to 12 oocytes (mean±SEM, representative of three similar experiments).

Fig 9. Localization of the human AE3 gene to chromosome 2 by polymerase chain reaction (PCR) analysis of human/hamster somatic cell hybrids. Genomic DNA from each of cell lines in the National Institute of General Medical Sciences human/rodent somatic cell hybrid mapping panel 2 and from hamster, mouse, and human cell lines was subjected to PCR amplification of the C1 exon of human AE3 as described in "Materials and Methods." The PCR reactions were fractionated on sequencing gels and subjected to $^{35}$S autoradiography. Lanes are labeled at top with the human chromosome uniquely carried by the particular hybrid cell line or by the species of the control cell line. The arrow at right marks the 135-nt AE3 PCR fragment. Size standards were from an M13 sequence ladder.
immunoblot. The C-terminal antibody did not detect bAE3 polypeptide, despite the presence of bAE3 mRNA in the tissue sample. We are currently developing antibodies specific for bAE3 to extend our search for the polypeptide product of the bAE3 transcript that is expressed in the heart.

Despite the expectation that AE3 is a glycoprotein, initial attempts to deglycosylate cardiac membrane cAE3 were unsuccessful, using procedures that quickly deglycosylated AE2 and AE1 (A. Zolotarev and S.L. Alper, unpublished data, 1994). In contrast, several other cardiac membrane polypeptides nonspecifically detected by secondary antibody alone were fully deglycosylated under all conditions tried. Human cAE3 overexpressed in CHO cells was also subjected to PNGase treatment in postnuclear membranes and in detergent extract and was similarly resistant to deglycosylation (not shown). It has been reported previously for the β subunit of the gastric H⁺,K⁺-ATPase that the high-mannose form of the glycoprotein is susceptible to PNGase F, whereas the low-mannose post-Golgi form cannot be deglycosylated by that enzyme.28 Polypeptides identified by antibody to the NHE2 Na⁺-H⁺ antiporter were also resistant to digestion by PNGase F.29 cRNAs encoding both bAE3 and cAE3 were injected into Xenopus oocytes, and both conferred increased 36Cl⁻ uptake when compared with water-injected oocytes. Recently, human bAE3-mediated Cl⁻-HCO₃⁻ exchange has also been demonstrated in transiently transfected CHO cells (L. Jiang, D. Yannoukakos, and S.L. Alper, unpublished data, 1993). The fold stimulation of 36Cl⁻ uptake into AE3 cRNA-injected oocytes was considerably lower than the ≥10- to 20-fold increases demonstrated by AE1 and AE2 cRNAs in our hands.20 This observation may correspond to the reported 75% lower transport activity of 293 cells overexpressing bAE3 compared with AE2.28 The possible requirement of a tissue-specific cofactor or chaperonin was tested by coinjection of human heart mRNA with cAE3 cRNA. Recombinant AE3-mediated 36Cl⁻ uptake was not enhanced, nor did the cardiac mRNA alone increase 36Cl⁻ uptake into oocytes (not shown). Although previous data have suggested no functional difference between bAE3 and AE2,29 more detailed comparative study may yet reveal functional or regulatory differences between them or between the two AE3 isoforms.

As the putative principal Cl⁻-HCO₃⁻ exchanger of heart, AE3 is a credible candidate gene for heritable or acquired disorders of cardiac rhythm, contractility, or morphogenesis and potentially for disorders involving other tissues that express AE3. To date, no such disorders have been mapped to human chromosome 2, nor have any corresponding heritable phenotypes in the mouse been linked to the corresponding murine AE3 locus.

Since only AE3 among the AE isotypes has been cloned from heart, it seems likely (but remains to be demonstrated) that AE3 mediates at least a part of the Cl⁻-HCO₃⁻ exchange in cardiac myocytes. Cl⁻-HCO₃⁻ exchange is present in Purkinje fibers and in isolated cardiac myocytes, where it is thought to participate in maintaining [Cl⁻] high above the predicted equilibrium value and to mediate a large portion of pHᵢ recovery from alkaline loading. Since inhibition of anion transport has been associated with arrhythmogenesis12 and acidic pHᵢ has been associated with protection of cardiocytes during recovery from in vitro ischemia,31 AE3-mediated Cl⁻-HCO₃⁻ exchange may serve an important role in normal cardiac function. Cl⁻-HCO₃⁻ exchange in cardiac Purkinje fibers has been reported to be activated by extracellular ATP, with properties suggesting mediation by a purinergic receptor.32 The presence in AE3 of a consensus nucleotide binding site allows a preliminary hypothesis that guanine nucleotides or metabolic energy state may mediate purinergic or metabotrophic regulation of AE3 function in some conditions.

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Molecular cloning, expression, and chromosomal localization of two isoforms of the AE3 anion exchanger from human heart.

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