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Structure and expression of subunit A from the bovine chromaffin cell vacuolar ATPase

Ying-Xian Pan[1], Jin Xu[2], Jane E. Strasser[2], Michael Howell[2] and Gary E. Dean[2]

[1]*Department of Physiology and Biophysics* and [2]*Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0524, USA*

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Subunit A of the vacuolar H^+-ATPase class is thought to be responsible for the ATP hydrolysis which drives proton-pumping. We report here the cloning and sequence determination of the first mammalian cDNA encoding a bovine vacuolar ATPase subunit A from an adrenal medulla cDNA library. Northern blots of bovine adrenal medulla RNA reveal a message of approximately 3.8 kb. The predicted peptide sequence, consisting of 618 amino acids with a calculated molecular weight of 68397 daltons, is similar to the sequences of the three known subunit A proteins. β-Galactosidase-subunit A fusion proteins were immuno-decorated by an antiserum raised to the subunit A protein from corn coleoptile vacuoles.

H^+-ATPase; Bovine adrenal medulla; cDNA cloning; Subunit structure

1. INTRODUCTION

Vacuolar proton-pumping adenosine triphosphatases (H^+-ATPases) serve to acidify certain intracellular compartments in eukaryotic cells, including secretory organelles, coated vesicles, endosomes, lysosomes, the trans-Golgi network, and the vacuoles of fungi and plants [1,2]: they serve to provide the motive force for a variety of trans-membrane transport processes and are responsible for ligand–receptor uncoupling. All of the H^+-ATPases purified to date are multi-subunit proteins which share similar structural features. The enzyme has an overall size of 450–750 kDa [3–5] and is composed of a trans-membrane proton pore (V_o) to which is attached a dissociable hydrophilic catalytic complex (V_1). This latter complex is composed of three copies each of a catalytic subunit A (~70 kDa)[5,6], a non-catalytic subunit B (~60 kDa) and one copy each of at least three other subunits [5,7]. We present here the cDNA sequence of the A subunit from the bovine chromaffin cell vacuolar ATPase and its predicted linear protein structure.

2. MATERIALS AND METHODS

2.1. *Materials*

A kind gift of bovine adrenal glands was obtained within 20 min of slaughter from Kluener Packaging Co., Cincinnati, OH; the medullae were promptly removed and flash frozen in liquid N_2. DNA modification and restriction enzymes were from New England Biolabs, Gibco-BRL, or Boehringer-Mannheim. Sequenase sequencing kits were purchased from US Biochemical Corp. Blotting media were from either Schleicher and Schuell (nitrocellulose), MSI (Nytran), or DuPont (GeneScreen Plus). All radiochemicals were from NEN. Oligonucleotide synthesis reagents were from ABN. All other reagents were reagent grade or better.

Correspondence address: G.E. Dean, Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0524, USA. Fax: (1) (513) 558 8474.

2.2. *cDNA cloning, sequencing, and sequence analysis*

Uni-directional, double-stranded cDNA was prepared from bovine chromaffin cell mRNA [8], the 1.5–4.5 kDa size fraction cloned into lambda ZAP [9], and the cDNA packaged to produce a library containing approximately 1×10^6 independent clones. Degenerate oligonucleotides designed to encode the predicted peptide sequence of the *Daucus carota* vacuolar ATPase subunit A at peptide positions 280–289 (sense orientation, GD2355: GGCGAGCTCGGNAA[C/T]GA[A/G]ATGGCNGA[A/G]GT, N=all four deoxynucleotide triphosphates) and 436–441 (anti-sense orientation, GD2356: CTTGGCCAG[C/T]TT[C/T]TT[A/G]TCNA[A/G]NCCCCA) were used in PCR reactions to amplify bovine subunit A cDNA; the template in each reaction was 10 ng of purified lambda ZAP library DNA. The PCR reaction products were sub-cloned into Bluescript SK(−) at the *Eco*RV site and the DNA sequences of the inserts were determined. A promising insert was in turn used to screen the lambda ZAP library by hybridization [8]. Subsequent to cloning, super-infection with VCSM13 helper bacteriophage was used to excise the Bluescript SK(−) plasmid containing inserts [9]. All DNA sequencing was performed by the dideoxy method as described [8] on double-stranded plasmid DNA. Computer analysis was performed using DNANALYZ (Gregory Wernke, University of Cincinnati), and Clone and Align software from Scientific and Educational Software. The bovine subunit A DNA sequence has been submitted to GenBank and has been given Accession Number X58386.

2.3. *Northern blot analysis*

Poly(A)-selected RNA was isolated from flash-frozen adrenal medullae as described [8]. RNA was denatured in 50.7% formamide and separated by electrophoresis through 1% agarose gels in 0.22 M formaldehyde. Capillary transfer to GeneScreen Plus membrane was performed for 12 h in a buffer containing 0.025 M $NaPO_4$, pH 6.5. The membrane was baked at 80°C for 2 h, pre-hybridized under standard

conditions [8], and then probed by incubation in the same buffer containing double-stranded DNA probe at approximately 10^6 cpm/ml, 10^8 cpm/μg. The probe was labeled with [^{32}P]dATP by PCR amplification [8].

2.4. *Immuno-blot analysis*

XL-1Blue cells carrying Bluescript SK(−) plasmids containing DNA fragments encoding various portions of the subunit A protein in frame with the *lacZ* α protein were grown at 37°C in LB medium to a density of ~3 × 10^8 cells/ml. Isopropyl thio-β-D-galactoside (IPTG) was added to a final concentration of 10 mM and the cells were incubated for an additional 2 h. The bacterial cultures were centrifuged (5 min, 12 600×*g*), the pellets resuspended and incubated for 10 min at 100°C in buffer containing 9 M urea, 10% SDS, and 5% 2-mercaptoethanol, and undissolved material sedimented by centrifugation (10 min, 12 600×*g*). The supernatants containing the fusion proteins were then separated by electrophoresis on 10% SDS-polyacrylamide gels [10]; replicas of these gels were electrophoretically transferred to nitrocellulose and the transferred blots probed with antisera raised in rabbits against corn coleoptile vacuolar subunit A [4]; ^{125}I-labeled goat anti-rabbit sera was used to decorate the immunoreactive proteins and the immunoreactive regions visualized by autoradiography.

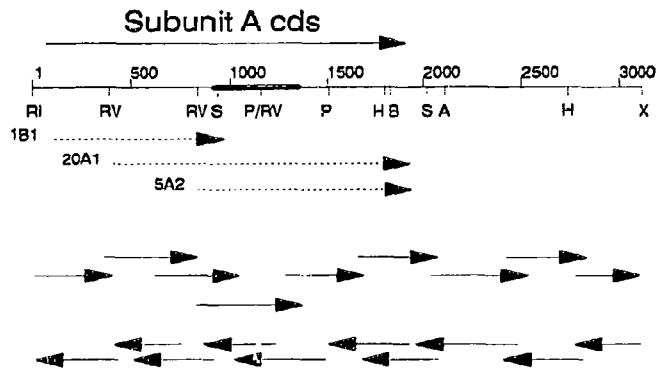

Fig. 2. Diagram showing the locations of the initial PCR fragment, sequencing strategy, and β-galactosidase fusion proteins used for Western blotting. The initial PCR fragment is indicated by a solid bar. Single-headed, dashed arrows labeled 1B1, 20A1, and 5A2 below the primary line indicate fusion protein fragments. Single-headed, solid arrows indicate the DNA fragments used for determining the DNA sequence. Abbreviated restriction enzyme sites: A, *Apa*I; B, *Bam*HI; H, *Hin*dIII; R, *Eco*RI; P, *Pst*I; S, *Sac*I; X, *Xho*I.

3. RESULTS

3.1. *Cloning and analysis of the bovine subunit A cDNA*

PCR amplification of DNA derived from the bovine adrenal medulla cDNA library with the degenerate oligonucleotide primers resulted in the production of a 492 bp DNA fragment which was sub-cloned into the Bluescript SK(−) vector. Northern blotting of this fragment against poly(A)-selected RNA from bovine chromaffin cells indicated that the subunit A mRNA was approximately 3.8 kb in length (Fig. 1). The amplified product was radio-labeled and used to probe 2.5×10^5 plaques from the same cDNA library at high stringency. Seventeen clones were isolated from this screening and checked for size; one of the longest clones (8B-b2) was selected for further analysis and its DNA insert sequence, presented in Fig. 3, was determined.

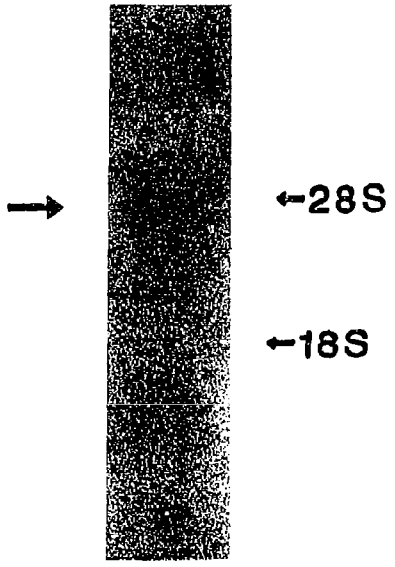

Fig. 1. Northern blot of bovine adrenal medulla mRNA with a PCR-generated Subunit A DNA fragment. Large arrow on the right indicates the 3.8 kb transcript. The probe was cDNA from subunit A cDNA nucleotide positions 939–1265.

The protein sequence is predicted to begin at nucleotide number 57 in the sequence, preceded by a termination codon closely upstream at position 48. The predicted start codon is immediately followed by a second methionine residue; while the second codon matches the consensus translational start sequence slightly better than the first, it is usually the case that the first codon in such a pair is used and we have thus chosen this codon with which to start the translation [11].

3.2. *Western blotting*

To confirm that the cloned DNA in fact encoded the subunit A protein, three fusion proteins carrying the indicated different portions of the protein (Fig. 2) were expressed in bacteria and reacted with a rabbit antiserum raised against the subunit A protein from corn coleoptile vacuoles [4]. As shown in Fig. 4 (lanes B–D), the antiserum reacted with the fusion proteins. Lane A, demonstrating no immunoreactivity, contains total protein extracted from a clone carrying the Bluescript plasmid with no insert.

4. DISCUSSION

The described cDNA resembles previously described vacuolar ATPase subunit A cDNAs and furthermore, antisera developed against a corn subunit A protein react with fusion proteins whose synthesis is programmed by this cDNA. We conclude that this cDNA encodes the bovine subunit A. Südhof et al. [12] have previously presented peptide sequence data for the bovine subunit A protein. There exists 95% agreement

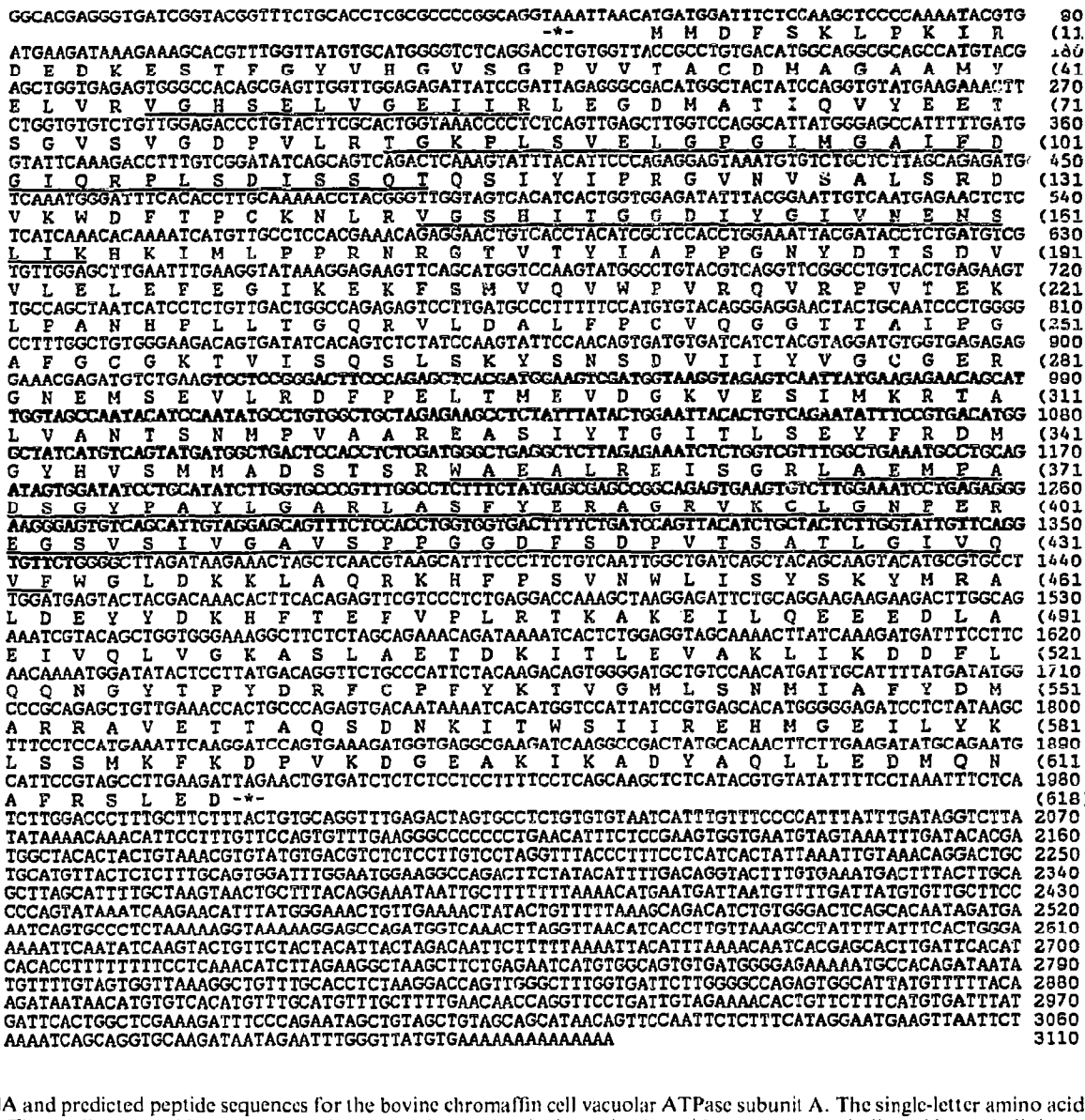

Fig. 3. cDNA and predicted peptide sequences for the bovine chromaffin cell vacuolar ATPase subunit A. The single-letter amino acid code is used throughout. The predicted peptide sequences which match the previously determined peptide sequences are indicated by underlining; discrepancies are indicated by italics. Also noted are the initial PCR product (bold-face) and the in-frame stop codon upstream of the predicted start site for translation.

between the cDNA-predicted peptide sequence and the peptide sequences themselves (129/136 residues). We suspect that the discrepancies between the cDNA-predicted peptide sequence and the peptide sequences are the result of mis-calls in the original peptide sequencing, for an examination of the other known subunit A sequences reveals an identity at nearly all of these positions between them and the cDNA-predicted peptide sequence. All of the discrepancies reside in only two tryptic peptides, T-233 and T-229 [12].It is possible, however, that these are allelic differences, or that there are two separate genes encoding subunit A proteins which behave essentially identically.

Comparison of the bovine subunit A protein sequence with the predicted subunit A protein sequences from other species indicates that there is overall identity with the carrot and *Neurospora*, and the mature form of the *Saccharomyces*, with predicted protein sequences of 68.6%, 63.1%, and 64.9%, respectively. It has been previously noted that the known subunit A protein sequences show identity with the protein sequences for the F1 β subunit (approximately 25% [13]). The underlined residues in Fig. 5 indicate identities between the sequences of all known subunit A proteins and the F_1 β proteins from both *E.coli* and *S. cerevisiae*, presumably residues critically important in enzyme structure or

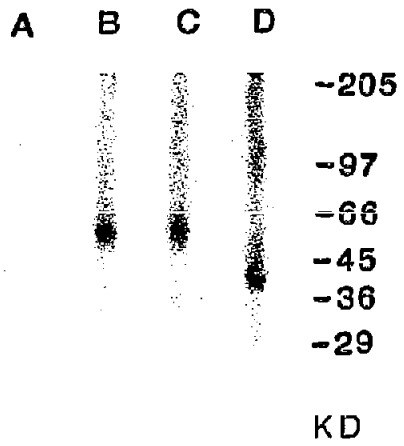

Fig. 4. Immuno-blots of portions of subunit A expressed as β-galactosidase fusion proteins with anti-subunit A antisera. XL-1 Blue bacterial lysates (approximately 10 μg per lane) carrying assorted Bluescript SK(−) plasmids. (Lane A) Bluescript SK(−), no insert; (lane B) clone 5A2, carrying residues 830–3110; (lane C) clone 20A1, carrying cDNA residues 410–3110; (lane D) clone 1B1, carrying residues 92–938.

function. From the pattern of substituted residues, we predict that several regions of the linear peptide sequence may be found to be exposed on the outer surface of the protein, namely the linear regions from 1–25, 109–118, 129–149, 172–192, 198–209, 294–306, 459–493, and 539–618. Further work is currently being conducted to determine whether these predictions are in fact correct.

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Bt  MMDFSKLPKIRDED--K--------ESTFGYVHGVSGPVVTACDMAGAAMYELVRVGHSELVGEIIRLEGDMATIQVYEETSGVSVGDPVL  ( 81)
Dc  .PSVYGDRLTTF..SE.--------..EY...RK.....V.DG.G.....DN.I.....S.....A.LM.N....  ( 83)
Sc    MAGAIENA.K.I--.RISLEDHA..EY.AIVS.....I.EN.I.C.....K...DN....V..ID..K.....A.LT.....  ( 87)
Nc      MAPQQNGA.V--D--------GIHT.KIVS.....V.E..I.V.....K...DQ....V..IN..Q.....A..M.....  ( 77)

Bt  RIGKPLSVELGPGIMGAIFDGIQRPLSDISSQTQSIYIPRGVNVSALSRDVKWDFTPCKNLRVGSHITGGDIYGIVNENSLIK-HKIMLPP  (171)
Dc  ..H.....L.N.....KI.AKRSGDV.....S.P..DK.TL.E.Q.-.KIGE.DLL....L.AT.F....MQ-.HVA...  (172)
Sc  .....L.ET.Y.....KA.KEES.....IDTP..D.TI..Q...G.-FQ..D..S.....S.F.....SS...L...  (177)
Nc  .....LLNN.Y.....EK.AEASN.....IATP..D.KK..E...--IMK..D..A...VW.T.Y...F.SV...L...  (166)

Bt  RNRGTVTYIAPPGNYDTSDVVLELEFEGIKEKFSMVQVWPVRQVRPVTEKLPANHPLLTGQRVLDALFPCVQGGTIAIPGAFGCGKTVISQ  (262)
Dc  DAM.KI..V..A.Q.SLK.T.....Q.V.KQ.T.L.T....TP...AS..A.DT.....S.L...C.....  (263)
Sc  .S...I.W...A.E.TLDEKI..V..D.K.SD.TLYHT....VP.....S.DY.....C.....  (268)
Nc  .A...I.R..EK.E.TVEEKI..V..D.K.TEYP.M.T....VP..AA..HS..Q.F.V.....S.....V.....  (257)

Bt  SLSKYSNSDVIIYVGCGERGNEMSEVLRDFPELTMEV-DGKVESIMKRTALVANTSNMPVAAREASIYTGITLSEYFRDMGYHVSMMADST  (352)
Dc  A.....TVV.....A...M...Q...TLP..RE..V....T.....IA.....N.....  (354)
Sc  .....A.....A...ME....YT.M-S.TK.P.....T.....A.....Q.KN...I...S  (358)
Nc  .V..F.....V.....A...K.....SI..-..RK.P.....T.I.....VA.....Q.MH.A.....S  (347)

Bt  SRWAEALRETSGRLAEMPADSGYPAYLGARLASFYERAGRVKCLGNPEREGSVSIVGAVSPPGGDFSDPVTSATLGIVQVFWGLDKKLAQR  (443)
Dc  .....A.....K.....G...N...T.....S.....  (445)
Sc  .....G.....Q.F.....K.....KAVA..S.D.T.....A....AD.....T.....T.....  (449)
Nc  .....G.....Q.F.....K.....K.QA..S.P.....  (438)

Bt  KHFPSVNWLISYSKYMRALDEYYDKHFTEFVPLRTKAKEILQEEEDLAEIVQLVGKASLAETDKITLEVAKLIKDDFLQQNGYTPYDRFCP  (534)
Dc  .....ST..ESF.E.FDSD.IDI....R.V..R.D..N.....DA.....I...LRE.Y.A..AF....K...  (536)
Sc  .....I.TSV.....TNV.NKF..SNYP..PV..DRM....SNA.E.EQV.....SA.SDS.....D..T...E.....ST..A...  (540)
Nc  .....I.TSV.....LTI..KW.EREYPD.PR..DRIRQL.SDS.E.DQV.....SA.SDP.....DM.T...E.....SD..Q...  (529)

Bt  FYKTVGMLSNMIAFYDMARRAVETTAQSDN-KITWSIIREHMGEILYKLSSMKFKDPVKDGEAKIKADYAQLLEDMQNAFRSLE---D  (618)
Dc  ...S.W.MR.N.H..NL.NQ...RG.GM.GQ..SYTL.KHRL.DLF.R.V.Q..E..-AE..DVLVGKFKK.HD.LTSG..N..DETR  (623)
Sc  IW..FD.MRAF.SYH.E.QK..------A.-GAN..KLADST.DVKHAV..S..FE.-SR..KEVHGEFEK..ST..ER.AEST---.  (607)
Nc  IW..EW.MKL.MG.H.E.QK.---I..GQ.----.NKV..ATQDLQAQ.K.L..EV.-SE.QE..CKK.EAIQQQ.LDK.A.VID--E  (617)
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Fig. 5. Comparisons of the bovine subunit A predicted protein sequence with subunit A protein sequences from other species. Bt, *Bos taurus*; Dc, *Daucus carota* (carrot); Sc, *Saccharomyces cerevisiae*; Nc, *Neurospora crassa*. Identities in the sequence are indicated by periods, differences by the indicated residue. Underlined residues are residues apparently shared with *Saccharomyces cerevisiae* F_1-β and *E. coli* F_1-β subunits.