MOLECULAR AND CELLULAR BIOLOGY, May 1989, p. 1978–1986
0270-7306/89/051978-09$02.00/0

Vol. 9, No. 5

Expression of Avian Ca^{2+}-ATPase in Cultured Mouse Myogenic Cells

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Received 7 November 1988/Accepted 7 February 1989

cDNA encoding Ca^{2+}-ATPase was cloned from a chicken skeletal muscle library. The cDNA (termed FCa) comprised 3,239 base pairs, including an open reading frame encoding 994 amino acids which showed the highest degree of homology with the adult rabbit fast-twitch Ca^{2+}-ATPase isoform (C. J. Brandl, S. de Leon, D. R. Martin, and D. H. MacLennan, J. Biol. Chem. 262:3768–3774, 1987). Radiolabeled FCa hybridized to a 3.2-kilobase transcript in chicken skeletal muscle RNA but not to cardiac muscle RNA, which confirmed its identity as encoding the fast Ca^{2+}-ATPase isoenzyme. FCa was transfected into the mouse myogenic line C2C12, from which a protein of 100 kilodaltons was immunopurified by using a monoclonal antibody specific for the avian fast Ca^{2+}-ATPase. Immunofluorescence microscopy of a line (designated C2FCa2) stably expressing the avian Ca^{2+}-ATPase localized the protein to the nuclear envelope and a population of cytoplasmic vesicles. A similar pattern was observed when C2FCa2 cells were stained with $DiOC_6(3)$, a cyanine dye that labels endoplasmic reticulum and mitochondria (M. Terasaki, J. Song, J. R. Wong, M. J. Weiss, and L. B. Chen, Cell 38:101–108, 1984). We conclude that the avian Ca^{2+}-ATPase fast isoform is expressed and correctly targeted to the endoplasmic reticulum in mouse C2C12 cells.

Ca^{2+}-ATPase plays a critical role in the physiology of contraction and relaxation of vertebrate skeletal muscle. This enzyme is an integral component of the sarcoplasmic reticulum (SR) membrane and actively transports calcium (released from the SR during muscle contraction) into the lumen of this organelle to elicit muscle relaxation. At least two isoforms are known to exist in mammals and birds (6, 19, 24). The fast isoform is expressed in fast-twitch skeletal muscle fibers, whereas the slow isoform is found in slow-twitch skeletal muscle fibers and cardiac muscle (6, 19). The cDNAs encoding the mammalian isoforms have been cloned (6, 24), as have cDNAs for neonatal and adult forms of the fast Ca^{2+}-ATPase, which have slightly different COOH termini generated in the rabbit via alternative splicing (5). Whereas the biochemical significance of the differences between the amino acid sequences of these forms is not clear, the structural differences between the fast and slow enzymes have allowed the production of isoform-specific antibodies (19, 50).

Although the structure and reaction mechanism of Ca^{2+}-ATPase have been extensively studied (25), many aspects of its biosynthesis and the mechanism of its retention in the SR (targeting) remain unknown. Ca^{2+}-ATPase is synthesized on membrane-bound polysomes (29) in a signal recognition particle-dependent manner (3) with an uncleaved signal sequence which remains to be identified (35). In muscle it is found in the SR, where it constitutes as much as 70% of the protein of this membrane (25). How the nascent Ca^{2+}-ATPase polypeptide is targeted to the rough endoplasmic reticulum (ER) and then to the SR, and what factors are involved in inserting such high levels of this enzyme into the SR membrane, are questions which are not yet answered. Targeting domains have recently been identified in proteins that reside in the ER, the probable nonmuscle homolog of the SR. Protein disulfide isomerase, grp94, and grp78, three luminal ER proteins, share a four-amino-acid sequence (KDEL) at their carboxy termini which is responsible for their retention in this organelle (30). E19, a transmembrane protein of the ER encoded by adenoviruses, has been reported to contain targeting information within a short sequence at the carboxy terminus which extends into the cytoplasm (31). The retention of rotavirus protein VP7 (34) and 3-hydroxy-3-methylglutaryl coenzyme A reductase (41) in the ER is dependent on the transmembrane domains of these proteins. Analogous sequences are likely to exist in Ca^{2+}-ATPase.

We have sought to approach these questions by devising a heterologous system in which avian Ca^{2+}-ATPase can be stably expressed and its biosynthesis and localization to the SR can be monitored by antibodies. This strategy has been used in analyses of other membrane proteins, including the acetylcholine receptor (28) and the subunits of Na^+,K^+-ATPase (44, 45), as well as many viral proteins (12, 36, 37, 48). An attractive possibility would be the transfer of avian Ca^{2+}-ATPase cDNAs into a mammalian myogenic cell line wherein both the endogenous and introduced Ca^{2+}-ATPase gene products could be detected by species-specific antibodies, allowing assessment of the effects of alterations in the introduced (avian) Ca^{2+}-ATPase against a background of unaltered endogenous (mammalian) enzyme. We report here the cloning of a cDNA encoding the entire fast isoform of avian Ca^{2+}-ATPase and its stable expression in mouse myocytes.

The mouse myogenic line C2C12 (4, 49) was chosen because we have generated monoclonal antibodies that bind mouse and chicken Ca^{2+}-ATPases with no detectable cross-reactivity. A cDNA encoding the fast Ca^{2+}-ATPase isoform, termed FCa, was constructed from clones isolated from an embryonic chicken skeletal muscle library and stably introduced into C2C12 cells (termed C2FCa2 cells). Immunofluorescence microscopy of dividing C2FCa2 cells, as displayed by an avian-specific antibody, revealed localization in

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the nuclear envelope and in membrane vesicles of varied sizes, indicative of an ER distribution.

MATERIALS AND METHODS

Cloning of the avian Ca^{2+}-ATPase cDNA. RNA was isolated from day 18 chicken embryo thigh muscle by guanidinium extraction (8) and selected twice by oligo(dT)-cellulose chromatography. Synthesis of cDNA by the method of Gubler and Hoffman (16) was followed by addition of *Eco*RI linkers and ligation into λgt10 (18). Packaged bacteriophage (Promega Packagene) were plated with *Escherichia coli* Q358 and screened without amplification.

Oligonucleotides were synthesized on the basis of the amino acid sequence of the rabbit Ca^{2+}-ATPase (1, 2), incorporating mixtures of bases at positions of ambiguity: TT(A/G)TT(A/G)TACTT(C/T)GT(C/T)AA (Asn-Asn-Met-Lys-Gln-Phe, from tryptic fragment B [1, 2]); and CT(A/G)TT(C/T)TT(C/T)TT(A/G)TAC(A/G)A (Asp-Lys-Lys-Ans-Met-Leu, from tryptic fragment A2 [1, 2]). These were ^{32}P labeled with polynucleotide kinase and used to probe chicken RNA separated in formaldehyde-agarose gels and blotted onto nitrocellulose. Both oligonucleotides hybridized only to a muscle-specific avian RNA species of 3.2 to 3.4 kilobases (kb) that was highly enriched in poly(A)$^+$ RNA (data not shown). Nitrocellulose replicas of the plated cDNA library were similarly probed with the labeled oligonucleotides. A positive clone containing a 2.6-kb insert, confirmed by sequencing (below) to encode a portion of Ca^{2+}-ATPase, was labeled by nick translation and used to rescreen the library.

Twenty-six clones (average length, 1.3 kb) were isolated, three of which, in sum, encoded the full-length Ca^{2+}-ATPase fast-isoform cDNA. A single cDNA (FCa) encoding the entire enzyme was constructed in plasmid pEMBL19$^+$ (10) via ligation of overlapping clones at common restriction sites. Clones 23 and 5, which contain the 3′ two-thirds [including the poly(A) tail] and 85% of the protein-coding region, respectively, were ligated at an *Eco*RI site. This construction was then ligated to clone 15 at an *Ava*II site to complete the 5′ end. The final construction was verified by sequencing and designated p19FCa.

For sequencing, cDNA fragments were subcloned into pEMBL vectors (10). Sequencing was performed by chain termination (39) of ^{35}S-labeled molecules generated by modified T7 DNA polymerase (Sequenase; United States Biochemical Corp.) (43).

Northern (RNA) blot analysis. Total RNA from skeletal (thigh) and cardiac muscle was isolated from day 18 chicken embryos by guanidinium extraction (8), separated on agarose-formaldehyde blots, transferred to nitrocellulose, and baked at 80°C in vacuo. Blots were probed with a 2-kb *Eco*RI fragment of the Ca^{2+}-ATPase cDNA which had been ^{32}P-labeled by nick translation. Hybridization proceeded overnight in aqueous solution (no formamide) containing 6× SSPE (0.9 M NaCl, 7.6 mM EDTA, 60 mM NaH_2PO_4 [pH 7.4]) at various temperatures, as indicated. The blots were washed twice for 15 min each in 2× SSPE at room temperature and then at 42°C in 0.2× SSPE for 2 h.

Cell culture and transfection. Primary myogenic cultures from day 12 chicken embryos were established and maintained as previously described (11). The mouse muscle cell line C2C12 (kindly provided by Helen M. Blau; 4, 49), was maintained as undifferentiated myoblasts in Dulbecco modified Eagle medium containing 20% fetal calf serum. Where indicated, the culture medium contained 10 mM sodium butyrate.

The Ca^{2+}-ATPase cDNA was inserted into the *Eco*RI site of the eucaryotic expression plasmid pSVDF (45), derived from pSV2CAT (14). The resulting shuttle vector, pSVFCa, was transfected into growing C2C12 myoblasts by calcium phosphate precipitation (15). For each 100-mm-diameter dish, 1 μg of *Pvu*I-linearized pSVFCa and 0.1 μg of *Bam*HI-linearized pSV2Neo (42) were added, followed by selection 24 h later in medium containing 400 μg of G418 (GIBCO Laboratories, Grand Island, N.Y.) per ml. Colonies of resistant cells were isolated and expanded 3 weeks later. Cells were screened for avian Ca^{2+}-ATPase expression by indirect immunofluorescence, using an avian-specific monoclonal antibody (see below). One such cell line, designated C2FCa2, which stably incorporated the avian Ca^{2+}-ATPase cDNA into its genome, was maintained under the same culture conditions as was the parent C2C12 cells and used in these experiments.

Monoclonal antibody production. Ca^{2+}-ATPase constitutes 60 to 70% of the protein in the SR. SR membranes were purified from adult chicken breast muscle and adult rabbit leg muscle by the method of Meissner (27) and used as an immunogen to generate monoclonal antibodies against Ca^{2+}-ATPase. The immunization schedule and screening methods have been described previously (19). Briefly, SR vesicles (~200 μg of protein) were injected into the peritoneum and then the tail veins of mice. Spleen cells were isolated, fused to myeloma cells, and hypoxanthine-aminopterin-thymidine selected. Clones were initially chosen on the basis of their secretion of antibodies that bound SR vesicles immobilized on microdilution plates. Anti-Ca^{2+}-ATPase antibodies were subsequently identified by the ability to recognize a 100-kilodalton (kDa) protein on Western blots (immunoblots). A variety of antibodies that cross-reacted between mammalian and avian Ca^{2+}-ATPase forms were isolated. However, one antibody from each preparation proved specific for either mammalian (CaF2-immunoglobulin G [IgG]) or avian (CaF3-IgG) enzyme, as indicated by immunofluorescence and Western blot analysis (data not shown). An antibody directed against the slow/cardiac muscle isoform of the chicken Ca^{2+}-ATPase (CaS/C1-IgG) was produced and selected as described previously (19).

Ca^{2+}-ATPase expression analysis. The analysis of fixed, permeabilized C2FCa2 cells by immunofluorescence (22) and immunopurification from cells grown in [^{35}S]methionine-containing medium (13) were performed as in previous studies and are described briefly in the figure legends. Protein was measured by the method of Lowry et al. (23).

C2FCa2 cells were stained with the fluorescent cyanine dye $DiOC_6(3)$ to demarcate the ER (46). Cells were fixed for 10 min in 0.25% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4), washed briefly in phosphate-buffered saline, and stained for 30 s in $DiOC_6(3)$ at 1 μg/ml.

RESULTS

cDNA cloning of chicken skeletal muscle Ca^{2+}-ATPase. Two oligonucleotides were synthesized (with appropriate third-base ambiguities) on the basis of the amino acid sequence of the rabbit fast Ca^{2+}-ATPase isoform (1, 2). Their specificities were tested by blot hybridization to embryonic chicken RNA, where they exhibited muscle-specific hybridization to a transcript of 3.2 to 3.4 kb (data not shown), the expected size for an mRNA encoding Ca^{2+}-ATPase (~100 kDa). A cDNA library was constructed from embryonic chicken skeletal muscle RNA and screened with the oligonucleotides, yielding a partial clone of 2.6 kb encoding the

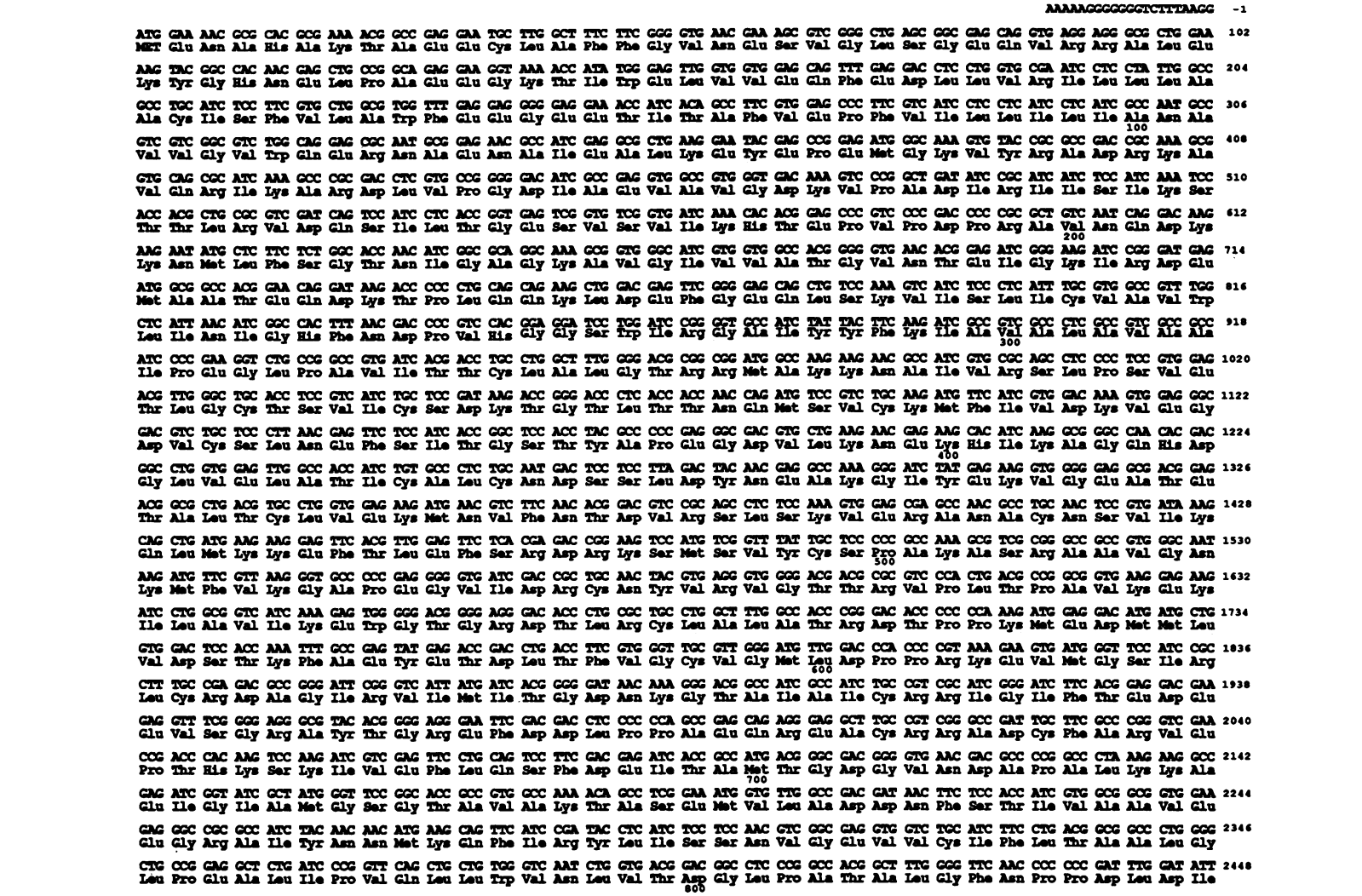

FIG. 1. Nucleotide sequence of FCa and deduced amino acid sequence of the protein product. Nucleotides are numbered in the 5′-to-3′ direction, beginning at the first base of the ATG codon of the initiator methionine. Negative numbers refer to the 5′ untranslated region. The signal for polyadenylation (underlined) was found at nucleotides 3144 to 3149.

Ca^{2+}-ATPase. The library was rescreened with this clone, which resulted in the isolation of 26 clones, none of which encoded the entire Ca^{2+}-ATPase. The complete Ca^{2+}-ATPase cDNA was constructed from three overlapping clones as described in Materials and Methods.

The Ca^{2+}-ATPase cDNA (FCa) comprised 3,259 base pairs (Fig. 1), including a region at the 3′ end corresponding to a tail of 70 adenines. This finding agreed with the ~3.2-kb transcript observed on RNA blots after hybridization with either oligonucleotides (data not shown) or fragments of FCa (see Fig. 3). The sequence of FCa revealed an open reading frame of 2,982 nucleotides beginning with the first ATG codon (residue 22), which resides in a consensus initiation sequence (20). After a TAA termination codon is 183 base pairs of 3′ untranslated sequence which contains the AATAAA polyadenylation signal 9 base pairs before the poly(A) tail.

Comparison of the nucleotide sequence of FCa (Fig. 2) with cDNAs encoding the rabbit Ca^{2+}-ATPases (5, 6, 24) indicated that the highest correlation was to the cDNA encoding the adult fast-twitch isoform. FCa and the cDNA encoding the adult rabbit fast Ca^{2+}-ATPase exhibited 82% identity, whereas the cDNA encoding the rabbit slow/cardiac Ca^{2+}-ATPase showed 74% similarity to the chicken

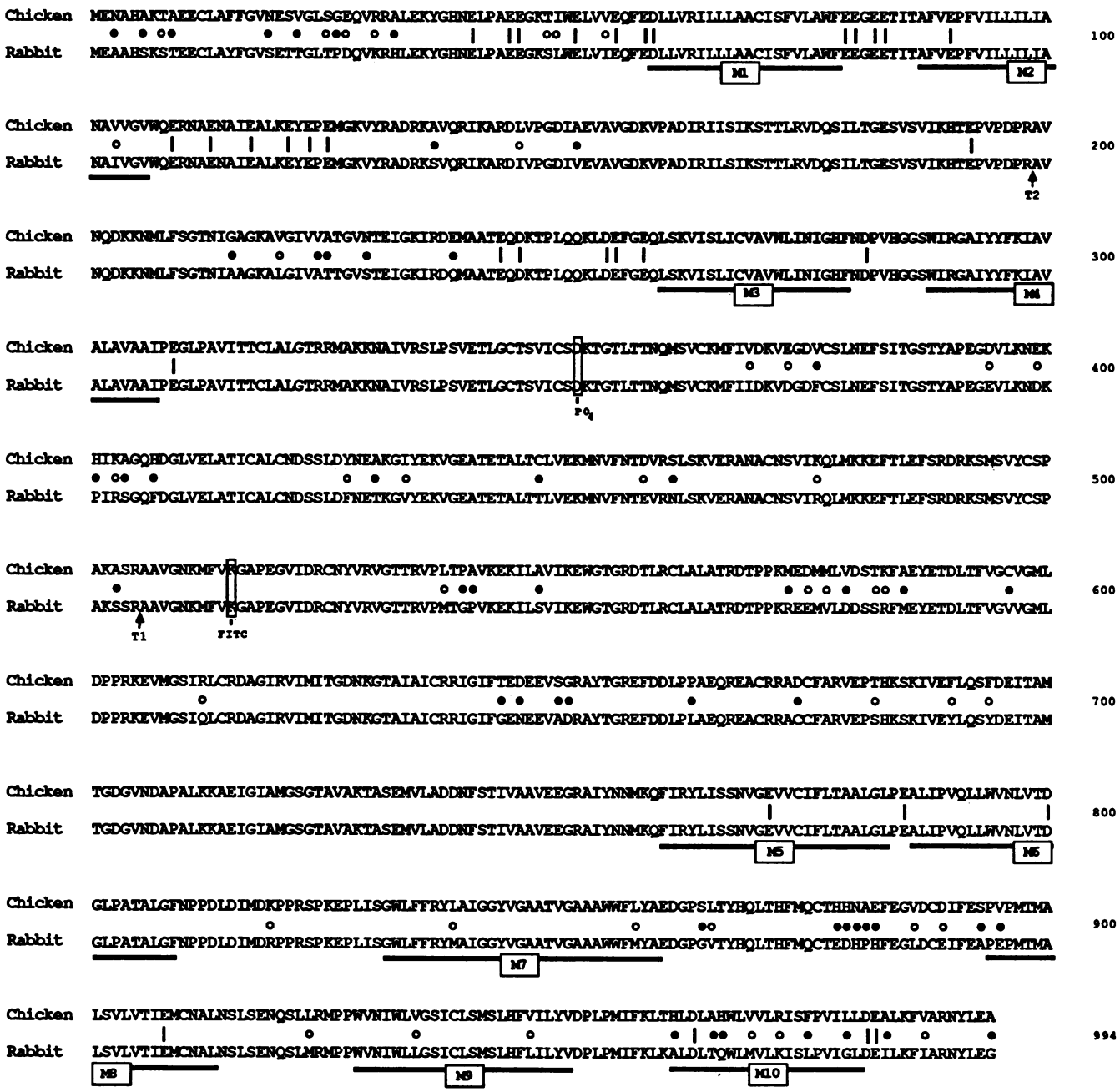

FIG. 2. Comparison of the deduced amino acid sequences of the chicken and adult rabbit fast Ca^{2+}-ATPases (5). The chicken and rabbit proteins are shown in one-letter code, beginning at the initiator methionine. The sites of phosphorylation (PO_4) and nucleotide (FITC) binding are boxed. Symbols: ▲, tryptic sites (T2 and T1); ○, conservative replacements; ●, nonconservative replacements. In accordance with the model of Brandl et al. (6), transmembrane regions (M1 to M10) are underlined, and acidic moieties proposed to participate in calcium binding and transduction are indicated by vertical bars.

cDNA. The deduced amino acid sequence of FCa also corresponded most closely with those of the rabbit fast Ca^{2+}-ATPase isoforms, showing 91% homology, with 43 of the 94 changes being conservative. The rabbit slow/cardiac enzyme shared 83% amino acid identity with the chicken Ca^{2+}-ATPase. The COOH terminus of the chicken Ca^{2+}-ATPase lacked the eight highly charged residues characteristic of the rabbit neonatal fast isoenzyme (6) and instead terminated in Ala, similar to the Gly of the adult rabbit fast form (5). Based on these criteria, we tentatively identified the protein product of FCa as the adult isoform of the avian fast Ca^{2+}-ATPase.

As expected from the primary structure similarity between the chicken and rabbit enzymes, domains identified as being involved in catalysis exhibited high conservation (Fig. 2). The phosphorylation sites and binding regions for the nucleotide analog fluorescein isothiocyanate (FITC) were identical, the latter further identifying FCa as encoding the fast Ca^{2+}-ATPase since, in the rabbit, the FITC-binding domain is one of the regions of highest divergence between the fast

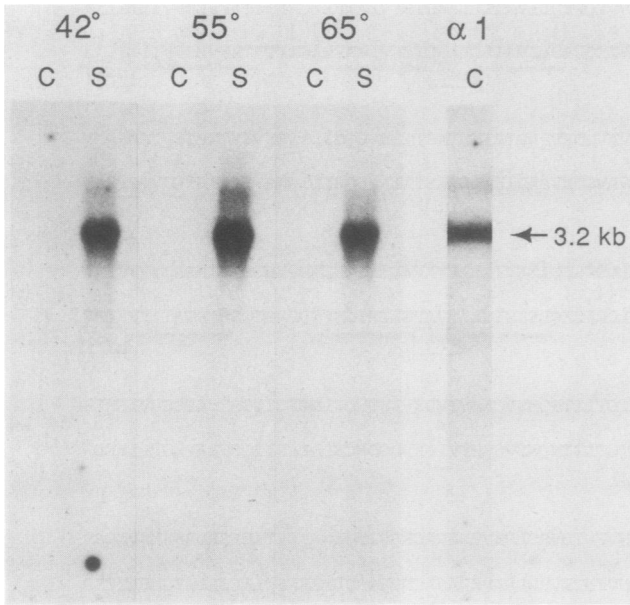

FIG. 3. Northern blot analysis. Total RNA was isolated by guanidinium extraction from skeletal (thigh) (S) and cardiac muscle (C) of day 18 chicken embryos, separated on formaldehyde-agarose gels (10 μg per lane), and blotted onto nitrocellulose. Identical blots were hybridized in aqueous (no formamide) solution at the temperature indicated with a 2-kb *Eco*RI fragment of pFCa which had been ^{32}P-labeled by nick translation; the blots were then washed as described in Materials and Methods. A band of ~3.2 kb (arrow), the expected size of the Ca^{2+}-ATPase transcript, is seen in lane S but was not found in the cardiac RNA at any stringency. To verify the integrity of the cardiac RNA, a lane from an identical blot was hybridized with a radiolabeled cDNA encoding the chicken Na^+,K^+-ATPase catalytic subunit (α1 isoform [44]). A transcript of ~3 kb was readily detected, which indicated that the RNA was intact.

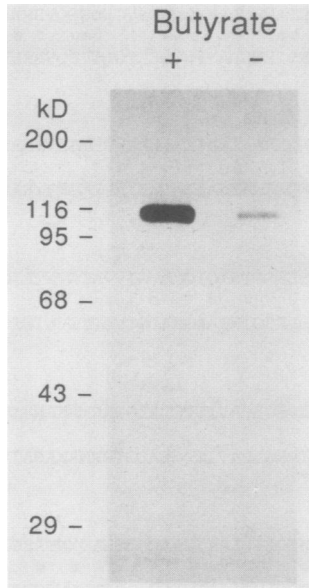

FIG. 4. Immunopurification of avian Ca^{2+}-ATPase from C2FCa2 cells. C2FCa2 cells were grown for 48 h with or without 10 mM sodium butyrate. During the final 16 h, the cells were maintained in methionine-free medium supplemented with 50 μCi of [^{35}S]methionine (Translabel; ICN Pharmaceuticals Inc., Irvine, Calif.) per ml and lysed in 1% Triton X-100; portions containing 450 μg of protein were incubated with CaF3-IgG-conjugated Sepharose beads. Antigen was eluted in sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer, separated on a 9% polyacrylamide gel, and fluorographed. Masses (in kilodaltons) of marker proteins are indicated at the left.

and slow/cardiac isoforms (6). FCa also closely resembled the rabbit fast Ca^{2+}-ATPase in the proposed calcium-binding region and in 9 of the 10 proposed transmembrane domains (6); the exception was M10, in which only 15 of the 22 residues were conserved. Significantly, all of the acidic amino acids hypothesized (6) to function in calcium binding and transduction that are conserved between rabbit isoforms were also present in the avian Ca^{2+}-ATPase.

Tertiary structure conservation between the two species was indicated by tryptic digestion of chicken Ca^{2+}-ATPase in situ, which yielded fragments identical in size to those generated with rabbit enzyme (19). Accordingly, the two tryptic cleavage sites identified in the mammalian enzymes (6, 24) are present in the deduced product of FCa at arginines 198 and 505.

Northern blot analysis. Comparison (see above) of the amino acid sequence deduced from FCa with those of the rabbit fast (5, 6) and slow/cardiac (24) Ca^{2+}-ATPases suggested that the avian cDNA encoded the fast isoform. Further evidence resulted from RNA blot analysis. Total RNA was isolated from cardiac and thigh muscle of day 18 chicken embryos, tissues which express slow/cardiac and primarily fast (as well as a small amount of slow/cardiac) Ca^{2+}-ATPase, respectively (19). A 2-kb *Eco*RI fragment of FCa encoding the 5′ two-thirds of the coding region hybridized solely to a thigh muscle transcript of ~3.2 kb; no hybridization to cardiac RNA was detected under a variety of stringencies (Fig. 3). The same result was obtained with a probe encoding the 3′ end of the FCa coding region (not shown). A 3-kb transcript was seen when cardiac RNA was probed with radiolabeled cDNA encoding the catalytic subunit of chicken Na^+,K^+-ATPase (Fig. 3, lane α1). Therefore, the absence of a detectable cardiac Ca^{2+}-ATPase transcript was unlikely to be the result of RNA degradation. These data support the conclusion that FCa encodes an avian fast Ca^{2+}-ATPase isoform.

Expression of FCa in mouse myogenic cells. FCa was transferred to the eucaryotic shuttle vector pSVDF, a modified form of pSV2CAT, which expresses from the simian virus 40 early promoter. The mouse myogenic cell line C2C12 was cotransfected with this construction and pSV2Neo by calcium phosphate precipitation and subjected to G418 selection. Resistant clones were screened for expression of avian Ca^{2+}-ATPase by monoclonal antibody binding (see below). Several positive lines which fluorescence microscopy indicated were expressing similar levels of avian Ca^{2+}-ATPase were isolated. One line was designated C2FCa2 and studied further.

Immunopurification of the protein product of the introduced avian cDNA is shown in Fig. 4. Detergent extracts were prepared from C2FCa2 cells grown in the presence of [^{35}S]methionine and incubated with CaF3-IgG, a monoclonal antibody specific for the avian fast Ca^{2+}-ATPase, which had been covalently coupled to Sepharose beads. After washing, the eluted material was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorographed. A protein of ~100 kDa, the expected mass of authentic Ca^{2+}-ATPase, was observed, the amount of which was increased in butyrate-treated cells (addition of sodium butyrate to the

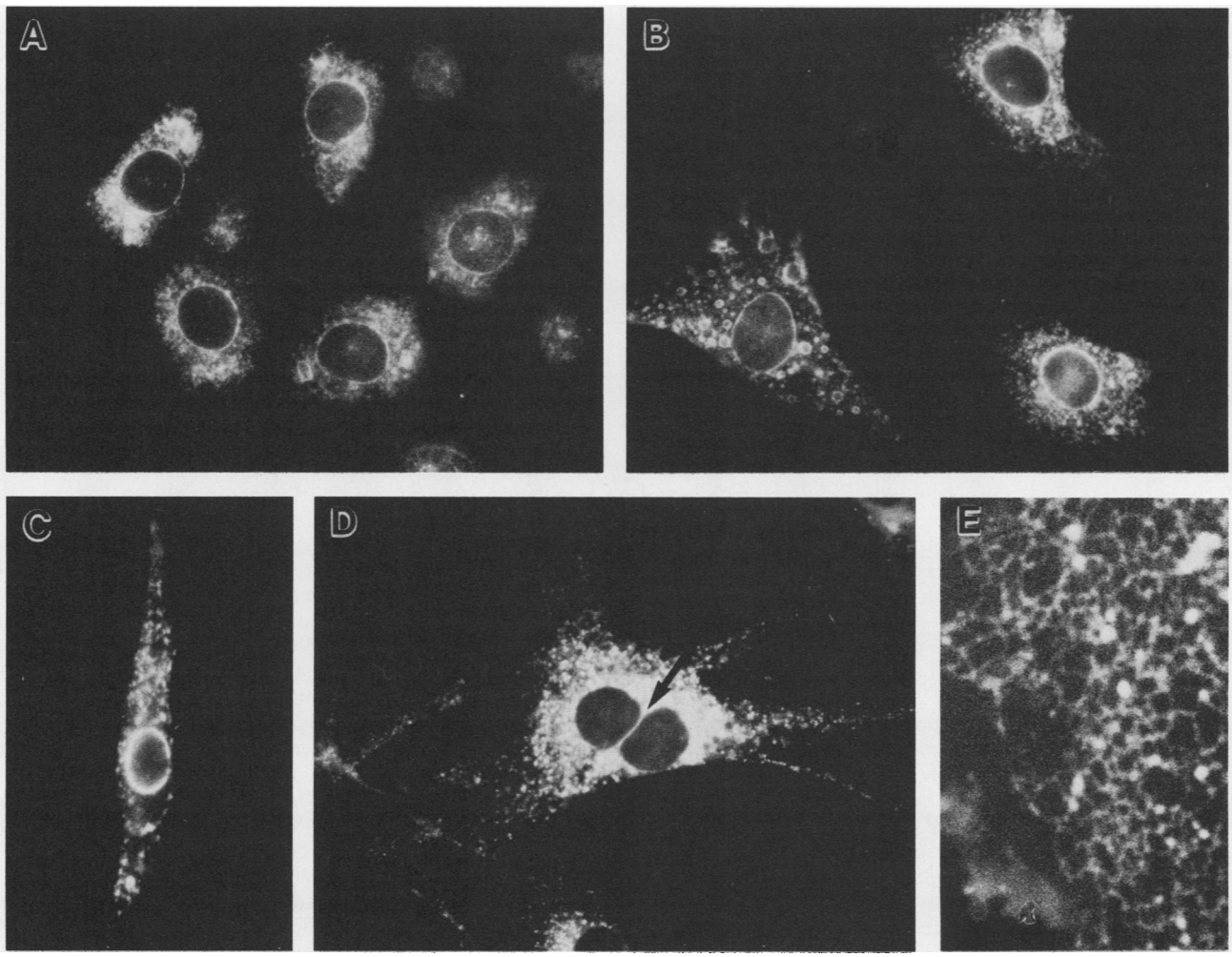

FIG. 5. Avian Ca^{2+}-ATPase expression in mouse C2C12 cells and primary chicken myocytes: correlation with ER distribution. (A and B) Growing C2C12 myoblasts were cotransfected with pSVNeo and pSVFCa and selected for the ability to grow in G418-containing medium. Cells expressing avian Ca^{2+}-ATPase, designated C2FCa2, were plated onto cover slips, lightly fixed in formaldehyde, and permeabilized with saponin. Binding of the CaF3-IgG was visualized with rhodamine-conjugated goat anti-mouse IgG. Before immunolabeling, cells were grown for 48 h in the absence (A) or presence (B) of 10 mM sodium butyrate. (C) Ca^{2+}-ATPase expression in chicken myocytes. Primary chicken myogenic cells were isolated from day 12 embryos and plated at sparse density in tissue culture. The cells were induced to differentiate without fusion by being maintained in mitogen-poor medium (Dulbecco modified Eagle medium plus 2% horse serum). Pictured is a chicken myocyte with the avian fast Ca^{2+}-ATPase isoform visualized as described above. (D) C2FCa2 cells labeled with $DiOC_6(3)$ dye. The cells were stained for 30 s at a dye concentration of 1 μg/ml, which labels the ER and mitochondria. The arrow indicates the nuclear envelope, seen most clearly in binucleate cells with closely apposed nuclei. (E) High-magnification image of the periphery a C2FCa2 cell stained with $DiOC_6(3)$ showing the netlike pattern characteristic of ER (30, 46).

culture medium increases expression of the transfected gene product [21]); butyrate treatment did not induce the appearance of any other proteins in the eluate.

C2FCa2 cells incubated with CaF3-IgG and fluorescent anti-mouse IgG revealed labeling of a heterogeneous population of cytoplasmic vesicles and of the nuclear envelope (Fig. 5A), the latter being characteristic of Ca^{2+}-ATPase in avian skeletal and cardiac muscle (19) as well as nonmuscle (Z. Kaprielian and D. M. Fambrough, manuscript submitted for publication) cells. Butyrate treatment of C2FCa2 cells enhanced expression of avian Ca^{2+}-ATPase (Fig. 4), but the general staining pattern was unchanged (Fig. 5B; butyrate causes C2FCa2 cells to flatten, increasing the resolution of fluorescence). No binding of CaS/C1-IgG, specific for the avian slow/cardiac Ca^{2+}-ATPase (19), was detected (not shown), in agreement with the assignment of FCa as encoding the fast Ca^{2+}-ATPase. Expression of the mouse fast isoenzyme in C2FCa2 cells, as determined by the binding of CaF2-IgG, was below the level of detection by immunofluorescence microscopy (not shown). Other isoforms of endogenous Ca^{2+}-ATPase for which we lack antibodies may exist.

Primary cultures of chicken myogenic cells differentiate in vitro by withdrawing from the cell cycle and fusing with adjacent myoblasts to form multinucleate syncytia (myotubes). A subset of cells differentiate, i.e., express proteins characteristic of the differentiated state, without fusing (myocytes), a phenomenon that can be induced by maintaining sparse cultures of myoblasts in medium containing cytochalasin B (38) or medium poor in growth factors (9 and references therein). In these myocytes, Ca^{2+}-ATPase expression was induced and the protein was routed to the nuclear envelope and cytoplasmic vesicles (Fig. 5C). This resembled the distribution of the enzyme in C2FCa2 cells

and suggested that the apparatus involved in localizing Ca^{2+}-ATPase in the mouse cells correctly situates the exogenous (chicken) enzyme.

The identity of the organelle to which the avian Ca^{2+}-ATPase is targeted was suggested by experiments using the fluorescent cyanine dye $DiOC_6(3)$, which, when used in high concentrations (0.5 to 2.0 μg/ml), labels the ER and mitochondria (46). C2FCa2 cells stained in this way (Fig. 5D) exhibited a pattern very similar to that seen with anti-avian Ca^{2+}-ATPase IgG (Fig. 5B), with label appearing on the nuclear envelope and membrane vesicles, as well as the characteristic meshwork of ER best visualized at the cell periphery (Fig. 5E). Mitochondria appeared as small, intensely stained structures that did not label with anti-Ca^{2+}-ATPase antibodies. Slight differences between the two staining patterns may have arisen because of the detergent permeabilization of the cells labeled with the antibodies. The broad fluorescence emission of $DiOC_6(3)$ precluded double labeling.

The mass (100 kDa), antibody-binding characteristics, and subcellular localization of the protein product of FCa lead us to conclude that the avian Ca^{2+}-ATPase is being expressed and that it contains the structural information necessary for targeting to the correct endomembranes in mouse myoblasts.

DISCUSSION

We have reported the isolation and expression in cultured myoblasts of a cDNA encoding an avian Ca^{2+}-ATPase. The assignment of the protein encoded in this cDNA as the fast-twitch fiber isoenzyme is based on several criteria: (i) hybridization of the cDNA to RNA from tissues expressing known isoforms, (ii) comparison of the deduced protein sequence with those of the mammalian Ca^{2+}-ATPases, and (iii) antigenic properties of the avian Ca^{2+}-ATPase expressed in transfected mouse cells.

The cDNA hybridized to RNA isolated from skeletal muscle (consisting mainly of fast-twitch fibers) but not at any stringency tested to cardiac RNA, which encodes the other known isoform, designated the slow/cardiac Ca^{2+}-ATPase (24). This result indicated that FCa encoded the fast Ca^{2+}-ATPase but also prevented its use as a probe for the slow/cardiac avian Ca^{2+}-ATPase. Avian isoforms may differ more than the mammalian counterparts, which cross-hybridized under conditions of moderate stringency (6).

Comparison of the deduced protein product of the avian cDNA with the rabbit Ca^{2+}-ATPases (5, 6, 24) indicated the highest degree of similarity to the adult fast isoenzyme, which shared amino acid identity at 91% of the residues (96% if conservative changes are not considered). Identity was especially high between the enzymes of the two species in the putative membrane-spanning domains with the exception of M10, which diverged at 7 of the 22 residues but differed in charge at only one position (Ala to His at the first amino acid of the M10 domain). The similarity of the chicken and rabbit Ca^{2+}-ATPases serves to support the structural model proposed by Brandl et al. (6). The domains involved in phosphorylation and nucleotide binding, as well as all of the acidic residues common to the mammalian fast and slow/cardiac enzymes that are postulated to participate in Ca^{2+} binding and transduction, are conserved in the avian Ca^{2+}-ATPase. Interestingly, the avian Ca^{2+}-ATPase resembled the mammalian adult isoform at the COOH terminus, the only site at which adult and neonatal enzymes of the rabbit differ, as a result of 3′ splicing of the encoding mRNAs (5). This was surprising in that FCa was obtained from a library constructed with late-embryo RNA. This finding may indicate that the avian neonatal-to-adult Ca^{2+}-ATPase transition occurs earlier than in mammals or that no such splicing event takes place in birds.

Recently, cDNAs encoding rabbit Ca^{2+}-ATPase isoforms were transiently expressed in COS-1 cells in an analysis of the catalytic domains of the enzyme (26). The subcellular distribution of the exogenous Ca^{2+}-ATPase was not determined. It was our aim to establish mammalian muscle cell lines stably expressing avian Ca^{2+}-ATPase as a means to study the biosynthesis and insertion of the enzyme into the SR. Mouse myogenic cells (C2C12 line) stably transfected with the chicken Ca^{2+}-ATPase cDNA (C2FCa2 cells) express a protein of ~100 kDa that is recognized by a monoclonal antibody specific for the avian fast isoform of this enzyme. The subcellular localization of this protein, as revealed by fluorescence microscopy of permeabilized cells, resembled that expected for the ER. Labeling was seen in a population of vesicles distributed evenly throughout the cytoplasm and in the nuclear envelope, generally considered an extension of the ER. A subset of the vesicles may represent calciosomes, discrete structures containing calsequestrin and postulated to function in inositol 1,4,5-triphosphate-sensitive calcium release (47). These organelles contain approximately 60% of the Ca^{2+}-pumping activity in nonmuscle cells. Since the C2FCa2 cells are undifferentiated, they may resemble nonmuscle cells in their distribution of Ca^{2+}-ATPase.

A similar pattern was seen when the cells were stained with the cyanine dye $DiOC_6(3)$, which labels the ER (and mitochondria) in a concentration-dependent manner (46). Colocalization of $DiOC_6(3)$ with fluorescently tagged anti-Ca^{2+}-ATPase IgG was not possible for two reasons. First, the treatment of cells with saponin, necessary to permeabilize them to the antibody molecules, abolishes the ability of the dye to intercalate into the ER (46). Second, the fluorescent emission of $DiOC_6(3)$ overlaps that of both rhodamine and fluorescein, the fluorochromes most commonly used for immunodetection. The use of cells permeabilized by nitrocellulose overlay (40) would alleviate the first problem but not the second.

The ER is homologous to the normal organelle in which this Ca^{2+}-ATPase isoform is resident, the SR. Since the C2FCa2 cells were undifferentiated, the ER would be the predicted target membrane of the Ca^{2+}-ATPase. Ca^{2+}-ATPase activity is associated with the ER of nonmuscle cells (17), although the structural properties of this enzyme in chicken tissues more closely resemble those of the slow/cardiac isoform (Kaprielian and Fambrough, submitted). Nuclear envelope labeling is observed in all tissues in which we can detect enzyme expression with anti-Ca^{2+}-ATPase IgGs (19). The significance of this is not clear, although this membrane is postulated as the site of synthesis of the crystalloid ER which is induced in CHO cells by 3-hydroxy-3-methylglutaryl coenzyme A reductase overexpression (32). The possibility exists that Ca^{2+}-ATPase is inserted first into the nuclear membrane and then distributed throughout the ER.

That the avian Ca^{2+}-ATPase appears in the ER of transfected mouse myoblasts suggests that targeting determinants within the enzyme are correctly recognized by the sorting apparatus of the mouse cells. Sequences that are necessary for retention in the ER have recently been described for several proteins. Four soluble proteins share a four-amino-acid sequence (KDEL) at their COOH termini, which is

sufficient to localize them in the ER and prevent their secretion (30). This short sequence also is functional when transferred to cathespin D, which normally is not found in this organelle (33). The mechanism by which proteins bearing this signal are retained in the ER appears not to involve anchoring (7) but rather to involve retrieval of the proteins after they exit the ER, probably from an early Golgi compartment (33). Targeting sequences have also been identified in integral membrane proteins that reside in the ER (31, 34, 41). In these cases, no consensus exists for the location of these signals within the proteins, as they are found variously in membrane-spanning regions (34, 41) and cytoplasmic tracts (31). Consequently, mechanisms by which membrane proteins are localized in the ER are yet to be defined.

None of the targeting sequences identified in other ER proteins appear in the avian Ca^{2+}-ATPase. Therefore, we seek to identify domains involved in targeting the Ca^{2+}-ATPase to the ER by transfecting mouse cells with systematically mutated cDNAs and monitoring the subcellular locations of the protein products. We had hoped that by transfecting the avian cDNA into a murine myogenic cell line, which would express an immunologically distinct (mouse) Ca^{2+}-ATPase upon differentiation in vitro, we would have a system in which two discernible Ca^{2+}-ATPases would be present; the endogenous enzyme could act as an internal marker of correct targeting. However, in contrast to the parent C2C12 cells, C2FCa2 cells do not express a high level of the mouse fast Ca^{2+}-ATPase when induced to differentiate in vitro (not shown). We are currently analyzing other independently isolated clones of transfected C2C12 cells to determine whether this is a general phenomenon. Regardless, the distinct pattern of expression of the avian Ca^{2+}-ATPase in mouse cells provides a convenient fingerprint for further studies to identify ER-targeting domains.

ACKNOWLEDGMENTS

This study was facilitated by the gift of C2C12 cells from Helen Blau. We are grateful to Axel Yup and Delores Somerville, who provided sound technical support throughout this project. We also thank Karen Renaud for critical review of the manuscript.

This study was supported by Public Health Service grant PO1-HL27867 (D.M.F.) and Public Health Service postdoctoral fellowship GM10939-02 (N.J.K.) from the National Institutes of Health.

LITERATURE CITED

1. **Allen, G., R. C. Bottomley, and B. J. Trinnaman.** 1980. The primary structure of the calcium ion-transporting adenosine triphosphatase from rabbit skeletal sarcoplasmic reticulum. Some peptic, thermolytic, tryptic and staphylococcal-proteinase peptides. Biochem. J. **187**:577–589.
2. **Allen, G., B. J. Trinnaman, and N. M. Green.** 1980. The primary structure of the calcium ion-transporting adenosine triphosphatase protein of rabbit skeletal sarcoplasmic reticulum. Peptides derived from digestion with cyanogen bromide, and the sequences of three long extramembranous segments. Biochem. J. **187**:591–616.
3. **Anderson, D. J., K. E. Mostov, and G. Blobel.** 1983. Mechanisms of integration of de novo-synthesized polypeptides into membranes: signal-recognition particle is required for integration into microsomal membranes of calcium ATPase and of lens MP26 but not of cytochrome b5. Proc. Natl. Acad. Sci. USA **80**:7249–7253.
4. **Blau, H., C.-P. Chiu, and C. Webster.** 1983. Cytoplasmic activation of human nuclear genes in stable heterocaryons. Cell **32**:1171–1180.
5. **Brandl, C. J., S. de Leon, D. R. Martin, and D. H. MacLennan.** 1987. Adult forms of the Ca^{2+}ATPase of sarcoplasmic reticulum. Expression in developing skeletal muscle. J. Biol. Chem. **262**:3768–3774.
6. **Brandl, C. J., N. M. Green, B. Korczak, and D. H. MacLennan.** 1986. Two Ca^{2+}ATPase genes: homologies and mechanistic implications of deduced amino acid sequences. Cell **44**:597–607.
7. **Cerioti, A., and A. Colman.** 1988. Binding to membrane proteins within the endoplasmic reticulum cannot explain the retention of the glucose-regulated protein GRP78 in Xenopus oocytes. EMBO J. **7**:633–638.
8. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
9. **Clegg, C. H., T. A. Linkhart, B. B. Olwin, and S. D. Hauschka.** 1987. Growth factor control of skeletal muscle differentiation: commitment to terminal differentiation occurs in G1 phase and is repressed by fibroblast growth factor. J. Cell Biol. **105**: 949–956.
10. **Dente, L., G. Cesareni, and R. Cortese.** 1983. pEMBL: a new family of single stranded plasmids. Nucleic Acids Res. **11**: 1645–1655.
11. **Devreotes, P. N., and D. M. Fambrough.** 1975. Acetylcholine receptor turnover in membranes of developing muscle fibers. J. Cell Biol. **65**:335–358.
12. **Doyle, C., M. G. Roth, J. Sambrook, and M.-J. Gething.** 1985. Mutations in the cytoplasmic domain of the influenza virus hemagglutinin affect different stages of intracellular transport. J. Cell Biol. **100**:704–714.
13. **Fambrough, D. M., and E. K. Bayne.** 1983. Multiple forms of $(Na^+ + K^+)$-ATPase in the chicken. Selective detection of the major nerve, skeletal muscle, and kidney form by a monoclonal antibody. J. Biol. Chem. **258**:3926–3935.
14. **Gorman, C. M., L. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
15. **Graham, F. L., and A. J. van der Eb.** 1973. A new method for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
16. **Gubler, U., and B. J. Hoffman.** 1983. A simple and very efficient method for generating cDNA libraries. Gene **25**:263–269.
17. **Heilman, K., C. Spamer, and W. Gerok.** 1984. The calcium pump in rat liver endoplasmic reticulum. Demonstration of the phosphorylated intermediate. J. Biol. Chem. **259**:11139–11144.
18. **Huyhn, T. V., R. A. Young, and R. W. Davis.** 1985. Constructing and screening cDNA libraries in λgt10 and λgt11, p. 49–78. *In* D. M. Glover (ed.), DNA cloning: a practical approach, vol. 1. IRL Press, Oxford.
19. **Kaprielian, Z., and D. M. Fambrough.** 1987. Expression of fast and slow isoforms of the Ca^{2+}-ATPase in developing chick skeletal muscle. Dev. Biol. **124**:490–503.
20. **Kozak, M.** 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44**:283–292.
21. **Kruh, J.** 1982. Effects of sodium butyrate, a new pharmacological agent on cells in culture. Mol. Cell. Biochem. **42**:65–82.
22. **Lippincott-Schwartz, J., and D. M. Fambrough.** 1986. Lysosomal membrane dynamics: structure and interorganeller movement of a major lysosomal membrane glycoprotein. J. Cell Biol. **102**:1593–1605.
23. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
24. **MacLennan, D. H., C. J. Brandl, B. Korczak, and N. M. Green.** 1985. Amino-acid sequence of a $Ca^{2+}+Mg^{2+}$-dependent ATPase from rabbit muscle sarcoplasmic reticulum, deduced from its complementary DNA sequence. Nature (London) **316**: 696–700.
25. **Martonosi, A. N., and T. J. Beeler.** 1983. Skeletal muscle, p. 417–485. *In* L. D. Peachy and R. H. Adrian (ed.), Handbook of physiology. American Physiological Society, Bethesda, Md.
26. **Maruyama, K., and D. H. MacLennan.** 1988. Mutation of aspartic acid-351, lysine-352, and lysine-515 alters the Ca^{2+} transport activity of the Ca^{2+}-ATPase expressed in COS-1 cells.

Proc. Natl. Acad. Sci. USA 85:3314–3318.
27. **Meissner, G.** 1975. Isolation and characterization of two types of sarcoplasmic reticulum vesicles. Biochim. Biophys. Acta **241**: 356–378.
28. **Mishina, M., T. Kurosaki, T. Tobimatsu, Y. Morimoto, M. Noda, T. Yamamoto, M. Terao, J. Lindstrom, T. Takahashi, M. Kuno, and S. Numa.** 1984. Expression of functional acetylcholine receptor from cloned cDNAs. Nature (London) **307**:604–608.
29. **Mostov, K. E., P. DeFoor, S. Fleischer, and G. Blobel.** 1981. Co-translational membrane integration of calcium pump protein without signal sequence cleavage. Nature (London) **292**:87–88.
30. **Munro, S., and H. R. B. Pelham.** 1987. A C-terminal signal prevents secretion of luminal ER proteins. Cell **48**:899–907.
31. **Paabo, S., B. M. Bhat, W. S. M. Wold, and P. A. Peterson.** 1987. A short sequence in the COOH-terminus makes an adenovirus membrane glycoprotein a resident of the endoplasmic reticulum. Cell **50**:311–317.
32. **Pathak, R. K., K. L. Luskey, and R. G. W. Anderson.** 1986. Biogenesis of the crystalloid endoplasmic reticulum in UT-1 cells: evidence that newly formed endoplasmic reticulum emerges from the nuclear envelope. J. Cell Biol. **102**:2158–2168.
33. **Pelham, H. R. B.** 1988. Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. EMBO J. **7**:913–918.
34. **Poruchynsky, M. S., C. Tyndall, G. W. Both, F. Sato, A. R. Bellamy, and P. H. Atkinson.** 1985. Deletions into an NH2-terminal hydrophobic domain result in secretion of rotavirus VP7, a resident endoplasmic reticulum membrane glycoprotein. J. Cell Biol. **101**:2199–2209.
35. **Reithmeier, R. A. F., S. de Leon, and D. H. MacLennan.** 1980. Assembly of the sarcoplasmic reticulum. Cell-free synthesis of the $Ca^{2+}+Mg^{2+}$-adenosine triphosphatase and calsequestrin. J. Biol. Chem. **255**:11839–11846.
36. **Roman, L. M., and H. Garoff.** 1986. Alteration of the cytoplasmic domain of the membrane-spanning glycoprotein p62 of Semliki forest virus does not affect its polar distribution in established lines of Madin-Darby canine kidney cells. J. Cell Biol. **103**:2607–2618.
37. **Rose, J. K., and J. E. Bergmann.** 1983. Altered cytoplasmic domains affect intracellular transport of the vesicular stomatitis virus glycoprotein. Cell **34**:513–524.
38. **Sanger, J. W.** 1974. The use of cytochalasin B to distinguish myoblasts from fibroblasts in cultures of developing chick striated muscle. Proc. Natl. Acad. Sci. USA **71**:3621–3625.
39. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
40. **Simons, K., and H. Virta.** 1987. Perforated MDCK cells support intracellular transport. EMBO J. **6**:2241–2247.
41. **Skalnik, D. G., H. Narita, C. Kent, and R. D. Simoni.** 1988. The membrane domain of 3-hydroxy-3-methylglutaryl-coenzyme A reductase confers endoplasmic reticulum localization and sterol-regulated degradation onto β-galactosidase. J. Biol. Chem. **263**:6836–6841.
42. **Southern, P., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
43. **Tabor, S., and C. C. Richardson.** 1987. DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc. Natl. Acad. Sci. USA **84**:4767–4771.
44. **Takeyasu, K., M. M. Tamkun, K. J. Renaud, and D. M. Fambrough.** 1988. Ouabain-sensitive $(Na^+ + K^+)$-ATPase activity expressed in mouse L cells by transfection with DNA encoding the α-subunit of an avian sodium pump. J. Biol. Chem. **263**:4347–4354.
45. **Takeyasu, K., M. M. Tamkun, N. R. Siegel, and D. M. Fambrough.** 1987. Expression of hybrid $(Na^+ + K^+)$-ATPase molecules after transfection of mouse Ltk^- cells with DNA encoding the β-subunit of an avian brain sodium pump. J. Biol. Chem. **262**:10733–10740.
46. **Terasaki, M., J. Song, J. R. Wong, M. J. Weiss, and L. B. Chen.** 1984. Localization of endoplasmic reticulum in living and glutaraldehyde-fixed cells with fluorescent dyes. Cell **38**:101–108.
47. **Volpe, P., K.-H. Krause, S. Hashimoto, F. Zorzato, T. Pozzan, J. Meldolesi, and D. P. Lew.** 1988. "Calciosome," a cytoplasmic organelle: the inositol 1,4,5-triphosphate-sensitive Ca^{2+} store of nonmuscle cells? Proc. Natl. Acad. Sci. USA **85**:1091–1095.
48. **Wills, J. W., R. V. Srinivas, and E. Hunter.** 1984. Mutations of the Rous sarcoma virus env gene that affect the transport and subcellular location of the glycoprotein products. J. Cell Biol. **99**:2011–2023.
49. **Yaffe, D., and O. Saxel.** 1977. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. Nature (London) **270**:725–727.
50. **Zubrzycka-Gaarn, E., G. MacDonald, L. Phillips, A. O. Jorgensen, and D. H. MacLennan.** 1984. Monoclonal antibodies to the $Ca^{2+}+Mg^{2+}$-dependent ATPase of sarcoplasmic reticulum identify polymorphic forms of the enzyme and indicate the presence in the enzyme of a classical high affinity Ca^{2+} binding site. J. Bioenerg. Biomembr. **16**:441–464.