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$(Ca^{2+} + Mg^{2+})$-dependent ATPase mRNA from smooth muscle sarcoplasmic reticulum differs from that in cardiac and fast skeletal muscles

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We have investigated some characteristics of the sarcoplasmic reticulum $(Ca^{2+} + Mg^{2+})$-dependent ATPase (Ca^{2+}-ATPase) mRNA from smooth muscle using specific cDNA probes isolated from a rat heart cDNA library. RNA blot analysis has shown that the Ca^{2+}-ATPase mRNA expressed in smooth muscle is identical in size to the cardiac mRNA but differs from that of fast skeletal muscle. S_1 nuclease mapping has moreover shown that the cardiac and smooth muscle isoforms possess different 3'-end sequences. These results indicate that a distinct sarcoplasmic reticulum Ca^{2+}-ATPase mRNA is present in smooth muscle.

Sarcoplasmic reticulum; $(Ca^{2+} + Mg^{2+})$-ATPase; RNA blot analysis; S_1 nuclease mapping; (Smooth muscle)

1. INTRODUCTION

The $(Ca^{2+} + Mg^{2+})$-dependent ATPase (Ca^{2+}-ATPase) of the sarcoplasmic reticulum (SR) is an ion-transport enzyme which, during muscle relaxation, transports Ca^{2+} liberated by the myofibrils from the cytosol to the lumen of the SR. The Ca^{2+}-ATPases of skeletal muscles have been extensively studied (review [1]). Two isoATPases were initially identified by immunological techniques [2,3] and more recently by cDNA cloning methods [4,5]. One of these isoforms is specific to fast skeletal muscle and the other, which is the product of a different gene, has been found in both cardiac and slow skeletal muscles [5,6]. These two isoforms may account for the different relaxation properties of the corresponding muscle types. Neonatal rabbit skeletal muscle contains another mRNA which is transcribed from the same gene as the adult fast muscle transcript but which shows alternative splicing of the 3'-end exon [6]. The corresponding neonatal protein has not as yet been isolated.

Much less is known about smooth muscles and their Ca^{2+}-ATPase. The ability of the SR from smooth muscle to accumulate Ca^{2+} was first shown by electron probe X-ray analysis in 1979 [7] and the dependence of Ca^{2+} uptake on ATP was subsequently demonstrated (review [8]). In smooth muscle a protein has been found which is similar to the Ca^{2+}-ATPase of skeletal or cardiac SR in its kinetic properties, molecular mass and tryptic digestion pattern but different according to its immunological properties [9,10]. In order to characterize the smooth muscle Ca^{2+}-ATPase with more precision we have studied some of the properties of its mRNA. By using cDNA probes specific to rat cardiac Ca^{2+}-ATPase we have shown that the SR Ca^{2+}-ATPase mRNA expressed in smooth muscle is similar in size to that expressed in cardiac muscle but different from that of fast skeletal muscle. Moreover, the cardiac and smooth muscle SR Ca^{2+}-ATPase mRNAs differ in their 3'-end sequence. These results indicate that in

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Abbreviations: Ca^{2+}-ATPase, $(Ca^{2+} + Mg^{2+})$-dependent ATPase; SR, sarcoplasmic reticulum

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smooth muscle a specific Ca^{2+}-ATPase mRNA is expressed.

2. MATERIALS AND METHODS

The cDNA probes to the Ca^{2+}-ATPase of rat heart were obtained by screening 400000 phages from an adult Sprague-Dawley rat heart cDNA library cloned in the *Eco*RI site of λgt11 (Clontech, RL 1006; 1.3×10^6 independent clones). The clones were hybridized with the 3′-end *Pst*I fragment from the rabbit slow/cardiac Ca^{2+}-ATPase cDNA (kindly provided by Dr D. MacLennan), which was labelled to a specific activity of $1-3 \times 10^9$ dpm/μg using the multiprime labelling kit (Amersham). 200 positive clones were obtained using the following high stringency washing conditions: 0.1 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0), 0.1% SDS at 65°C for 1 h. The sequence derived from these clones and which covers 50% of the mRNA sequence revealed 90% homology with the rabbit slow/cardiac isoform in the coding region and about 70% homology in the untranslated regions (De la Bastie et al., in preparation). Three probes were used in the present study and are represented in fig.1. Probe A is a 2.2 kb *Eco*RI fragment containing the 3′-end of the mRNA from nucleotide +1449 to the poly(A^+) tail. Probe B is a 1.5 kb *Eco*RI fragment from the 5′-end of the mRNA, nucleotide −50 to +1448. Probe C is a 1.1 kb *Eco*RI-*Pst*I fragment containing the entire 3′-untranslated region and about 350 bp of coding sequence.

The expression of the Ca^{2+}-ATPase mRNA was studied by RNA blot analysis and S_1 nuclease mapping using total RNA extracted from liver, cardiac ventricle, fast skeletal muscle (tensor fasciae latae, TFL) and smooth muscle (intestine, Int, and uterus, Ut) of adult Sprague-Dawley rats. After dissection the tissues were immediately frozen in liquid nitrogen and the RNA was isolated by the method of Chirgwin et al. [11]. For RNA blot analysis, total RNA was size-fractionated on 1% agarose gels containing formaldehyde and blotted onto nylon membranes (Pall Biodyne TM-A transfer membranes). The filters were prehybridized in 50% formamide, 0.1% bovine serum albumin, 0.1% ficoll, 0.1% polyvinylpyrrolidone, 0.05 M sodium phosphate buffer, pH 6.5, 0.1% SDS, 250 μg/ml salmon sperm DNA, at 42°C and hybridized using the same conditions with either probe A or B labelled to a specific activity of $1-3 \times 10^9$ dpm/μg. The filters were washed in 1 × SSC, 0.1% SDS at 38°C. For S_1 nuclease mapping analysis, 10 μg total RNA were hybridized with an excess of cDNA probe in 80% formamide. Probes A and C were used for these experiments. Probe A was labelled at the *Eco*RI site by filling-in using Klenow enzyme (Boehringer Mannheim), and the double-stranded probe was denatured before hybridization to the RNAs. The sense strand of probe C was inserted into M13mp19 and was used to ensure antisense probe, uniformly labelled by extension from the universal M13 primer using Klenow enzyme. The labelled fragment was isolated on a 6% sequencing gel. The RNA-cDNA hybrids were digested with S_1 nuclease (Amersham or New England Biolabs, 5 U/μg ARN) at 25°C and the resulting fragments separated on 6% sequencing gels (see [12]).

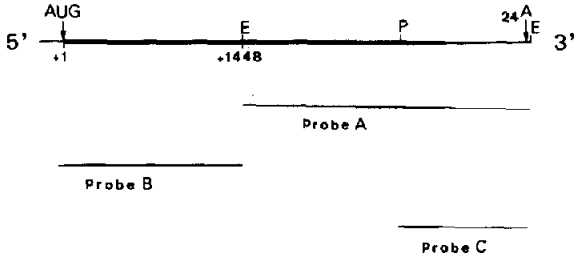

Fig.1. Schematic representation of the rat heart sarcoplasmic reticulum Ca^{2+}-ATPase mRNA and of the cDNA probes used in this study. The thick line corresponds to the coding sequence and the thin line to the untranslated regions. AUG, initiation codon; 24A, poly(A^+) tail; E, *Eco*RI; P, *Pst*I.

3. RESULTS AND DISCUSSION

Blots of total RNA from liver, cardiac, fast skeletal and smooth muscles were hybridized to probe A (fig.2). No hybridization was observed with liver RNA. In the striated muscle samples two sizes of Ca^{2+}-ATPase mRNA were detected: one of about 3.9–4 kb in cardiac muscle (fig.2, Vent) and another of 3.7 kb in fast skeletal muscle (fig.2, TFL). This is in full agreement with the sizes reported in rabbit muscles by Brandl et al. [4]. In the smooth muscle samples (fig.2, Int and Ut) there was clear hybridization with a 3.9 kb mRNA,

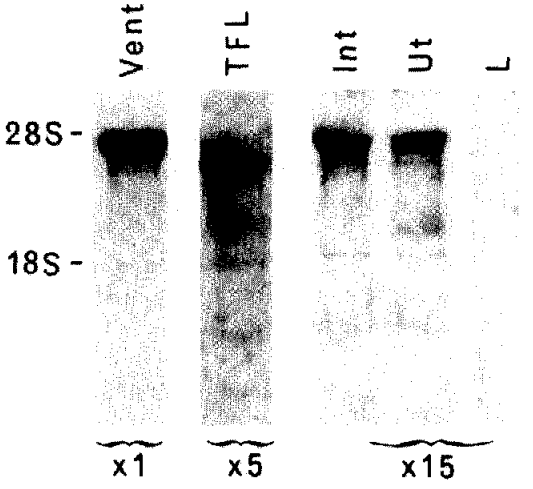

Fig.2. RNA blot analysis of total RNA from liver (L), cardiac (Vent), fast skeletal (TFL), and smooth (intestine Int, and uterus Ut) muscles, hybridized with probe A corresponding to the 3′-end half of the rat heart Ca^{2+}-ATPase mRNA. 5 μg total RNA from heart ventricle and 15 μg total RNA from the other muscles were loaded per lane. The blot was washed in 1 × SSC, 0.1% SDS at 38°C and exposed to autoradiography for 5 (× 1), 25 (× 5) and 75 (× 15) h at −70°C.

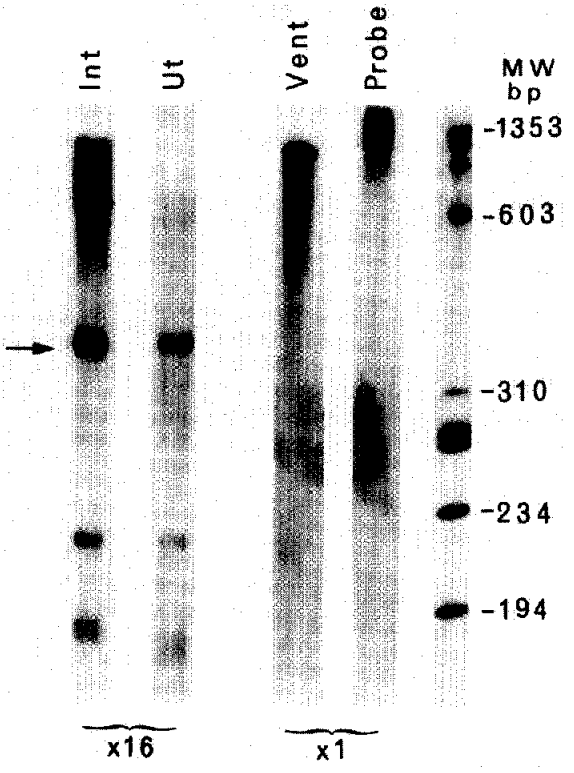

Fig.3. S_1 nuclease mapping analysis of RNA from cardiac muscle (Vent), intestine (Int) and uterus (Ut). 10 μg total RNA were hybridized with a uniformly labelled *PstI-EcoRI* cDNA fragment from the 3'-end of the rat heart Ca^{2+}-ATPase mRNA (probe C). Molecular mass markers are from ϕx174 digested with *HaeIII* and labelled at the 5'-end using T_4 polynucleotide kinase. The untreated probe is shown on the fourth lane. The gel was exposed to autoradiography for 1 h for the cardiac sample and the probe (× 1), and 16 h for the smooth muscle samples (× 16).

showing that the Ca^{2+}-ATPase mRNA expressed in these muscles is similar in size to that expressed in heart. Identical results were obtained with probe B (not shown). Thus smooth muscle mRNA may be either identical to that expressed in the heart or it may only be similar in size but different in sequence.

In order to explore this possibility S_1 nuclease mapping experiments were performed. We first used probe C uniformly labelled and these results are shown in fig.3. As expected when RNA from heart was hybridized to the probe, a protected fragment a little smaller than the probe itself was observed. The small difference in size is due to the fact that the poly(A^+) tail and the M13 primer which are included in the probe are not protected by the RNA. When RNAs from uterus or intestine were hybridized to probe C (fig.3, Ut and Int) no fragment of that size was detected. However, a partially protected fragment, 350 bp long, was clearly observed, indicating that while having the same size the cardiac and smooth muscle Ca^{2+}-ATPase mRNAs differ in their sequence. Since only one band is characteristic for the smooth muscle, the difference between the probe and the RNA is probably not due to a single difference. The smooth muscle mRNA differs from the probe by approx. 700 bp, which corresponds to the size of the untranslated region. The two additional bands of lower molecular mass (fig.3, Ut and Int) were also found to be present in the cardiac sample when the gel was exposed for the same length of

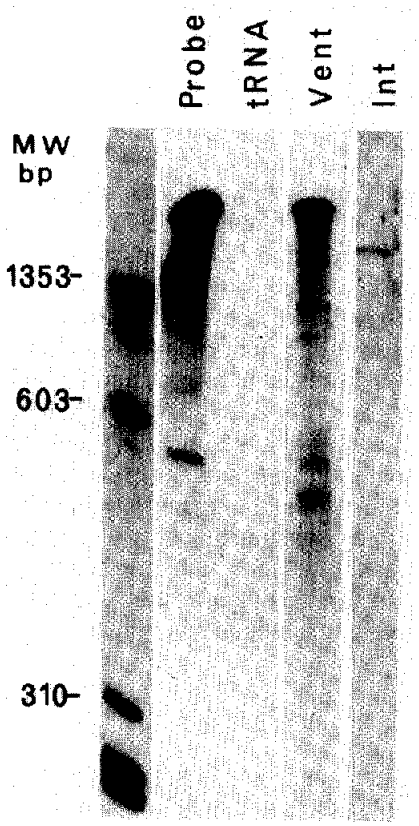

Fig.4. S_1 nuclease mapping analysis of *E. coli* tRNA and of RNA from cardiac ventricle (Vent) and intestine (Int). 10 μg of total RNA were hybridized with a 3'-end-labelled double-stranded 2.2 kb *EcoRI* fragment from the 3'-end half of the message (probe A). The gel was exposed to autoradiography for 96 h. The same molecular mass markers were used as in fig.3.

time as for the smooth muscle samples (not shown). However, the exact significance of these bands is as yet unclear. It is most likely that they are the result of the degradation of the single stranded probe during hybridization. It could also be suggested that they represent minor but as yet unidentified isoforms that are common to all muscles.

To further localize the divergence between cardiac and smooth muscle mRNAs we hybridized them to probe A, 3′-end-labelled at the *Eco*RI site (fig.4). In our hybridization conditions there was no reannealing of the two strands of the probe since no radioactive band was detected when *E. coli* tRNA (fig.4, tRNA) was used instead of muscle RNA. The probe is fully protected from S_1 nuclease digestion by RNA from cardiac muscle (fig.4, Vent) but is only partially protected by RNA from intestine (fig.4, Int). Label was located at the 3′-end of the strand complementary to the mRNA, this indicates that the smooth muscle Ca^{2+}-ATPase mRNA differs from that expressed in cardiac muscle by approx. 700 bp at its 3′-end, i.e. over the length of the 3′-non-coding region. Additional bands were observed but they were also found when the probe alone was run on the gel (fig.4, probe) and thus probably represented degradation of the probe.

Our results indicate that (i) the Ca^{2+}-ATPase mRNA expressed in fast skeletal muscle differs from that expressed in cardiac and smooth muscles by its size and (ii) the Ca^{2+}-ATPase mRNA present in cardiac and smooth muscle while identical in size when examined by RNA blot analysis, differs in sequence in the 3′-untranslated region. This is the first demonstration of the existence of a Ca^{2+}-ATPase mRNA different from those encoding the fast and slow cardiac isoforms. We cannot as yet conclude if the cardiac and smooth muscle Ca^{2+}-ATPase mRNAs differ only by their 3′-untranslated region or if differences extend into the coding region. Additional experiments are required to resolve this question and to determine whether the smooth muscle Ca^{2+}-ATPase is produced by splicing from one of the two genes already identified [4] or whether it is transcribed from another gene.

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