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Rabbit cardiac and slow-twitch muscle express the same phospholamban gene

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The nucleotide sequences of cDNAs encoding phospholamban were found to be virtually identical when the cDNA clones were isolated from rabbit slow-twitch (soleus) and rabbit cardiac muscle libraries. These findings demonstrate that both types of muscle express the same phospholamban gene. The deduced amino acid sequences of rabbit and dog phospholamban were identical except for a change from Asp (dog) to Glu (rabbit) at position 2. The nucleotide sequences of the 5'- and the very long 3'-untranslated regions of rabbit and dog phospholamban cDNAs also exhibited a high percentage of identity.

Phospholamban; Slow-twitch muscle; cDNA cloning; (Cardiac muscle)

1. INTRODUCTION

Phospholamban is the major phosphorylated protein in cardiac muscle sarcoplasmic reticulum following β-adrenergic stimulation of heart muscle. It is considered to be a regulator of the cardiac Ca^{2+}-ATPase [1,2], thereby mediating the effects of β-adrenergic agonists on sarcoplasmic reticulum function. Dog cardiac phospholamban has been sequenced [3,4] and a cDNA has been cloned [5]. The presence of phospholamban in slow-twitch muscle has been inferred from phosphorylation patterns and immunological cross reactivity [6,7], but it is not known whether this protein is identical to cardiac phospholamban. In this communication we report the molecular cloning of cDNAs for rabbit cardiac and slow-twitch muscle phospholamban and demonstrate that they are products of the same gene.

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The nucleotide sequence presented here has been submitted to the EMBL/GenBank database under the accession number Y00761

2. MATERIALS AND METHODS

2.1. *cDNA synthesis*

The synthesis of cDNA, its ligation into phage vectors and its amplification to form cDNA libraries were accomplished essentially as described by Brandl et al. [8] and Huynh et al. [9]. Cardiac cDNA was size-fractionated by 0.8% agarose gel electrophoresis, and the 1.5–4 kb fraction was used to construct the library in λgt10 arms (Stratagene Cloning Systems). An aliquot of the unamplified library was used for screening. The amplified slow-twitch muscle cDNA library, constructed in λgt11, was kindly provided by Dr L. Fliegel.

2.2. *Screening of the libraries*

All cDNA probes were labelled to high specific activity with a nick translation kit (Boehringer Mannheim). Hybridization was carried out in 50% deionized formamide, 5 × SSCP, 10 × Denhardt's solution, 0.1% SDS and 100 μg/ml heat-denatured salmon sperm DNA at 42°C overnight. The filters carrying the cDNA libraries were washed twice for 30 min in 2 × SSCP and twice for 30 min in 0.2 × SSCP and 0.1% SDS at 42°C. Northern blots were washed twice for 30 min in 2 × SSCP and twice for 30 min in 0.1 × SSCP and 0.1% SDS at 65°C.

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2.3. *DNA sequencing*

The cDNA inserts were excised by *Eco*RI digestion and subcloned into the Bluescript vector (Stratagene). Fragments overlapping the internal *Eco*RI sites were obtained by *Hin*dIII and *Bam*HI digestion. The nucleotide sequence was determined according to the strategy shown in fig.1 by the dideoxy sequencing method of Sanger et al. [10].

All other procedures were conducted according to standard methods [11].

3. RESULTS

The λgt10 cDNA library constructed from rabbit cardiac muscle mRNA was screened with a cDNA probe encoding dog cardiac muscle phospholamban (*Sac*I(−72)/*Dde*I(258)) [5]. Twelve clones to which the probe hybridized were isolated from about 10^5 plaques. Fig.1a shows a partial restriction map of the longest clone labelled RCP3. Nucleotide sequence analysis revealed that the insert contained an open reading frame of 156 bp that encoded rabbit cardiac phospholamban (fig.2). The amino acid sequence corresponded to dog cardiac phospholamban with the exception that Asp^2 in the dog protein [5] was replaced with Glu in the rabbit sequence. Dot matrix analysis of the rabbit cardiac muscle phospholamban and the longest dog cardiac muscle clone [12] indicated high nucleotide sequence identity, not only in the protein coding region (91%), but also in the 5′- and 3′-noncoding regions (not shown). There was, however, a gap in the similarity plot at 1.7–2.1 kb of the rabbit cDNA sequence suggesting a deletion in the dog phospholamban transcript in this region.

In order to confirm the presence of phospholamban in slow-twitch muscle, a Northern blot of poly(A) RNA from rabbit cardiac, slow-twitch and fast-twitch muscle was probed under conditions of high stringency with a fragment of RCP3 including

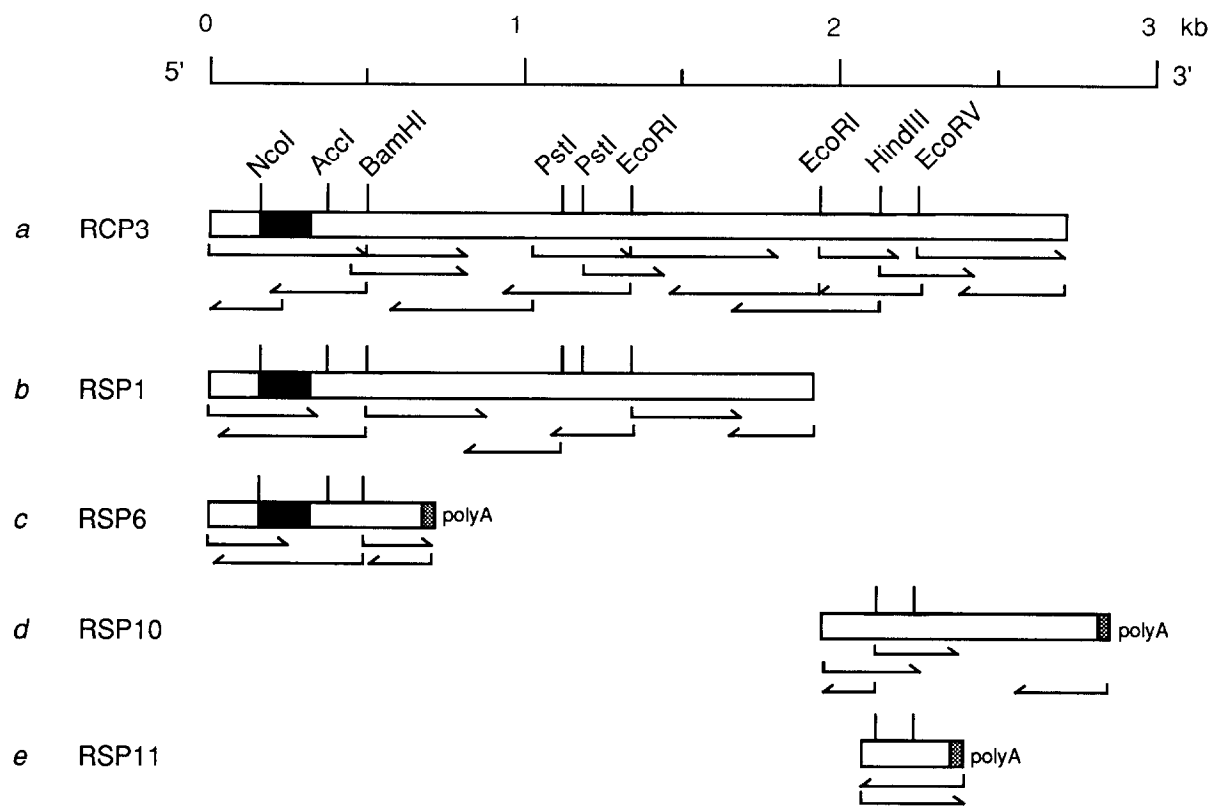

Fig.1. Partial restriction map and sequencing strategy of phospholamban cDNA clones from rabbit cardiac (RCP3) and slow-twitch (RSP1, RSP6, RSP10 and RSP11) muscles. The closed boxes indicate the coding region. RSP6, RSP10 and RSP11 have poly(A) tails at their 3′-ends.

Fig.2. Nucleotide and deduced amino acid sequences of rabbit cardiac and slow-twitch muscle phospholamban cDNA. The sequences of nucleotides −177 to −173, −172 to 2532 and 2533 to 2664 were from RSP6, RCP3 and RSP10, respectively. The oblique lines indicate the location of different polyadenylation sites in the cDNA sequences. The boxes indicate the presumed polyadenylation signals.

the entire coding region (5′-*Eco*RI linker-*Bam*HI(330)). Fig.3 shows that relatively high levels of a 3.4 kb transcript were found in both slow-twitch and cardiac muscle, but not in fast-twitch muscle mRNAs. Upon prolonged exposure of the autoradiograph (not shown), we observed faint bands of length shorter than 3.4 kb.

A λgt11 cDNA library constructed from slow-twitch muscle mRNA was screened, first, with a probe containing the coding region of rabbit cardiac muscle phospholamban (*Nco*I(1)/*Bam*HI-(330)) and, second, with a noncoding region probe from RCP3 (*Eco*RI(1742)/*Eco*RV(2176)). The lengths and restriction maps of four overlapping

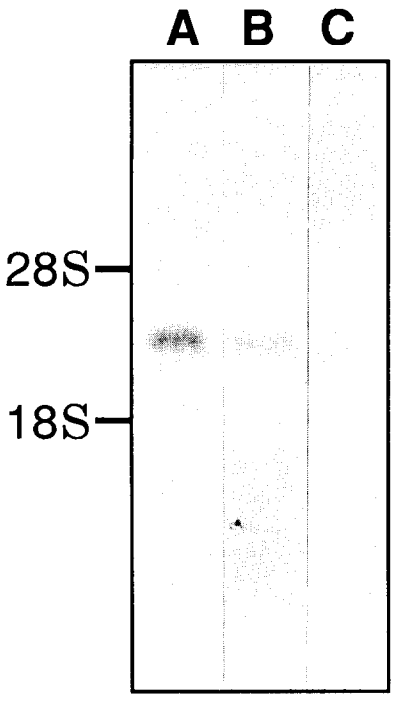

Fig.3. Northern blot analysis using 5 μg of poly(A) RNA from cardiac (lane A), slow-twitch (lane B) and fast-twitch (lane C) muscles. The blot was hybridized with a 439 bp cDNA fragment from rabbit cardiac phospholamban (5′-*Eco*RI linker-*Bam*HI (330) in fig.2). The positions of the 28 S and 18 S ribosomal RNAs are indicated.

clones isolated from the slow-twitch library are illustrated in fig.1. The map and the nucleotide sequence of the longest cDNA insert labelled RSP1 were exactly the same as nucleotides −172–1740 of RCP3 except that an additional 5 nucleotides were found at the 5′-end of the clone and that G(42) was substituted by A. Although this change is within the coding region, it is silent at the amino acid level. Similarly the sequences of clones RSP6, RSP10 and RSP11 were identical to their comparable sequences in RCP3.

4. DISCUSSION

We have isolated cDNA clones encoding phospholamban from both rabbit cardiac and slow-twitch muscle cDNAs libraries. The nucleotide sequences of these cDNAs are identical except for a single nucleotide which may reflect allelic variation between rabbits. Thus, both muscles express the same phospholamban gene. Since slow-twitch muscle expresses the same phospholamban gene and the same Ca^{2+}-ATPase gene [13] as cardiac muscle, it is likely that the Ca^{2+} pump is regulated in a similar fashion in both tissues.

A comparison of the deduced amino acid sequences for dog and rabbit phospholamban shows that the aspartic acid residue at position 2 in dog phospholamban [5] has been replaced by glutamic acid in rabbit phospholamban. A negative charge in this position may be sufficient for interaction of phospholamban with either dog or rabbit Ca^{2+}-ATPases, but it is also possible that there is a compensating change in the Ca^{2+}-ATPase sequence between dog and rabbit.

While the Northern blot of both cardiac and slow-twitch muscle mRNAs demonstrated that the phospholamban transcript is 3.4 kb long, we were unsuccessful in isolating a cDNA clone of this length. Moreover, we noted considerable heterogeneity in the use of polyadenylation signals in the cDNAs which we did isolate. Thus RCP3 and RSP1 were 2704 and 1917 bases long, respectively, but lacked poly(A) tails. Clones RSP6, RSP10 and RSP11 were all polyadenylated but at different sites from one another. A combination of RSP1 and RSP10 would span 2.8 kb, slightly longer than RCP3, leaving 0.6 kb of transcript sequence unaccounted for. In the light of the heterogeneity in the use of polyadenylation signals that we have observed in our cDNA library, we do not know whether this additional sequence is located in the 5′- or 3′-untranslated regions of the original transcript.

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REFERENCES

[1] Tada, M. and Katz, A.M. (1982) Annu. Rev. Physiol. 44, 401–423.
[2] Tada, M., Kirchberger, M.A. and Katz, A.M. (1975) J. Biol. Chem. 250, 2640–2647.
[3] Fujii, J., Kadoma, M., Tada, M., Toda, H. and

Sakiyama, F. (1986) Biochem. Biophys. Res. Commun. 138, 1044–1050.
[4] Simmerman, H.K.B., Collins, J.H., Theibert, J.L., Wegener, A.D. and Jones, L.R. (1986) J. Biol. Chem. 261, 13333–13341.
[5] Fujii, J., Ueno, A., Kitano, K., Tanaka, S., Kadoma, M. and Tada, M. (1987) J. Clin. Invest. 79, 301–304.
[6] Kirchberger, M.A. and Tada, M. (1976) J. Biol. Chem. 251, 725–729.
[7] Jorgensen, A.O. and Jones, L.R. (1986) J. Biol. Chem. 261, 3775–3781.
[8] Brandl, C.J., Fliegel, L. and MacLennan, D.H. (1987) Methods Enzymol., in press.
[9] Huynh, T.V., Young, R.A. and Davis, R.W. (1985) in: DNA Cloning. A Practical Approach (Grover, D.M. ed.) vol.1, pp.49–78, IRL Press, Oxford.
[10] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[11] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY.
[12] Uyeda, A., Kitano, K., Fujii, J., Kadoma, M., Tada, M. and Tanaka, S. (1987) Nucleic Acids Res. 15, 6738.
[13] Brandl, C.J., DeLeon, S., Martin, D.R. and MacLennan, D.H. (1987) J. Biol. Chem. 262, 3768–3774.