Structure of the Rabbit Phospholamban Gene, Cloning of the Human cDNA, and Assignment of the Gene to Human Chromosome 6*

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We have isolated and characterized genomic DNA clones encoding rabbit phospholamban. Only a single gene for phospholamban was detected in the rabbit genome. The phospholamban gene of 13.2 kilobases contains only one 10.5-kilobase intron, which separates exonic sequences located in the 5'-untranslated region. Two potential transcription initiation sites were mapped to 335 and 185 nucleotides upstream from the translation initiation site in the mRNA or 239 and 89 nucleotides upstream from the exon/intron boundary. Phospholamban gene expression in various smooth muscle tissues, as well as in cardiac and slow twitch skeletal muscle, was detected by Northern blotting. The phospholamban gene was mapped to human chromosome 6, using a human phospholamban cDNA.

Phospholamban, a pentameric protein composed of polypeptide subunits 52 amino acids in length, is a major substrate of the Ca<sup>2+</sup>-ATPase in the unphosphorylated state, but inhibition is relieved upon phosphorylation of the protein (Tada et al., 1988). The subsequent activation of the Ca<sup>2+</sup>-pump leads to enhanced muscle relaxation rates, thereby contributing to the inotropic response elicited in heart by β-agonists.

Phospholamban is expressed in slow twitch skeletal muscle (Kirchberger and Tada, 1976; Jorgensen and Jones, 1986; Fujii et al., 1988) and some smooth muscle cells (Raeymaekers and Jones, 1986; Ferguson et al., 1988; Verboom and Jones, 1989), as well as in cardiac muscle. It is not found in fast twitch skeletal muscle, however (Kirchberger and Tada, 1976; Jorgensen and Jones, 1986; Fujii et al., 1988). While cardiac

and slow twitch muscles express the same Ca<sup>2+</sup>-ATPase isoform (SERCA2a) (Brandi et al., 1986, 1987), smooth muscle expresses the product of an alternatively spliced transcript from the same gene (SERCA2b) (Lyttton et al., 1989). It is assumed that phospholamban regulates the activity of both the SERCA2 isoforms in these different tissues. The amounts of phospholamban and Ca<sup>2+</sup>-ATPase protein present at different developmental stages in sheep cardiac muscle were closely correlated (Mahony and Jones, 1986). Nevertheless, the expression of phospholamban and cardiac/slow twitch muscle Ca<sup>2+</sup>-ATPase genes do not seem to be coordinated in cardiac muscle tissues undergoing hypertrophy (Nagai et al., 1989).

In earlier studies (Fuji et al., 1987, 1988), we isolated cDNA clones encoding phospholamban. We found a long 3'-untranslated sequence with different polyadenylation sites in these clones. It was, therefore, of interest to investigate whether the gene structure might provide clues to the reason for the production of heterogenous mRNAs. In this paper, we describe the isolation and characterization of the rabbit phospholamban gene and its expression in various tissues. We also describe the isolation of human phospholamban cDNA and the mapping of the human phospholamban gene to human chromosome 6.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases and DNA-modifying enzymes were obtained from Boehringer Mannheim, Bethesda Research Laboratories, and Pharmacia LKB Biotechnology Inc. The Bluescript plasmid vectors were from Stratagene. [γ-<sup>32</sup>P]ATP, [α-<sup>32</sup>P]dCTP, and [α-<sup>32</sup>S]dATP were purchased from ICN Biomedicals or Amerham Corp. Oligodeoxyribonucleotides used for primer extension analyses were synthesized at Osaka University. All other chemicals were of the highest grade available.

Isolation of Genomic Clones—A rabbit genomic library constructed in λ phage Charon 4A (Maniatis et al., 1978) was a kind gift from Dr. T. Maniatis, Harvard University. We screened this library by recombinational screening (Seed, 1983) and hybridization screening. For recombinational screening, a Sau3AI-BamHI rabbit phospholamban cDNA fragment (residues 178–330; Fujii et al., 1988) was ligated into the miniplasmid λAβ7 and used as described previously (Korcek et al., 1988). For hybridization screening, we used a 255-bp AUS fragment (residues 56 to 199) containing the entire coding sequence and a 33-bp AUS-3′UTR fragment (residues −160 to −121) from the 5′-untranslated sequence, both of which were 5'-labeled by a random hexamer priming method (Feinberg and Vogelstein, 1983) using an oligolabeling kit (Pharmacia). Prehybridization and hybridization were carried out in 50% deionized formamide, 10 × Denhardt's

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1 The abbreviations used are: bp, base pair; SDS, sodium dodecyl sulfae; kb, kilobase(s); [α-<sup>32</sup>S]dATP, deoxyadenosine 5′-α-[<sup>32</sup>S]thiotriphosphate.
Fig. 1. Structure and restriction map of rabbit phospholamban genomic clones. The three upper lines represent the location of the overlapping genomic clones λrPG1 (17.0 kb), λrPG2 (19.4 kb), and λrPG3 (16.6 kb), spanning a total length of about 35 kb of genomic DNA. The fourth line shows the partial restriction endonuclease map of the gene derived from the three overlapping clones. Restriction endonuclease sites are: E, EcoRI; B, BamHI; H, HindIII. The fifth line shows the locations, determined by DNA sequence analysis, of the two exons within the gene, as solid boxes and the location of the coding sequence within Exon 2 as a white box. The positions of the CCAAT sequence and the ATG initiation codon are marked. The sixth line represents the mature mRNA, showing the three different poly(A) attachment sites identified by cDNA cloning (Fujii et al., 1988).

Fig. 2. Nucleotide sequences at the 5’ and 3’ ends of the phospholamban gene. The DNA sequence of the nontranscribed strand of the gene is displayed in the 5’ to 3’ direction. Nucleotide position +1 is assigned to the TATA box (CCAAT box). A transcription initiation site, determined by primer extension analysis using a primer complementary to the 31 residues underlined near the 5’ end of Exon 1, is indicated as constituting the 5’ end of exon 1. A second potential transcription initiation site lies 150 bases upstream of this site. Amino acids encoded within the second exon are labeled in the three-letter code beneath each codon. The protein sequence begins 21 bases upstream from the polyadenylation site near the 3’ end of the gene. The DNA sequence of the nontranscribed strand of the gene is displayed in the 5’ to 3’ direction. Nucleotide position +1 is assigned to the TATA box (CCAAT box). A transcription initiation site, determined by primer extension analysis using a primer complementary to the 31 residues underlined near the 5’ end of Exon 1, is indicated as constituting the 5’ end of exon 1. A second potential transcription initiation site lies 150 bases upstream of this site. Amino acids encoded within the second exon are labeled in the three-letter code beneath each codon. The protein sequence begins 21 bases upstream from the polyadenylation site near the 3’ end of the gene.
solution (1 × Denhardt’s; 0.02% (w/v) Ficoll 400, 0.02% (w/v) poly-vinylpyrrolidone, 0.02% (w/v) bovine serum albumin, fraction V), 1 × SSCP (120 mM sodium chloride, 15 mM sodium citrate, 13 mM potassium phosphate, and 1 mM EDTA, pH 7.2), 0.1 mg/ml salmon sperm DNA, and 0.1% SDS at 42 °C. Filters were washed twice for 30 min in 2 × SSCP and then twice in 0.1 × SSCP in the presence of 0.1% SDS at 65 °C. When the shorter probe was used, filters were hybridized under the conditions described above, except that 30% instead of 50% formamide was used and the filters were washed twice in 2 × SSCP, then twice in 1 × SSCP in the presence of 0.1% SDS at 42 °C. Large scale phage DNA preparations were made according to Maniatis et al. (1982).

Isolation of Rabbit Smooth Muscle and Human Phospholamban cDNA Clones—A rabbit uterus smooth muscle cDNA library constructed in λZAP (Stratagene) (Lytton et al., 1989) was screened with a 331-bp NcoI-BamHI fragment (residues -1 to 330) of rabbit cardiac muscle phospholamban cDNA. The human skeletal muscle cDNA library constructed in Agt10 (Koenig et al., 1987), a kind gift from Dr. L. M. Kunkel, Harvard University, was screened using a mixture of dog and rabbit phospholamban cDNAs as probes. After hybridization with 32P-labeled probes, filters were washed twice in 0.1% SDS and twice in 1 × SSCP at 42 °C in the presence of 0.1% SDS and were then subjected to autoradiography.

Genomic Southern Blot Analysis—High molecular weight genomic DNA was isolated from rabbit cardiac muscle and kidney tissues according to Maniatis et al. (1978). After digestion with appropriate restriction endonucleases, 20 pg of DNA was size-fractionated in 0.8% agarose gel. The fragments were transferred to nitrocellulose paper according to Southern (1975) and hybridized under the same conditions that were used for screening the library with the 255-bp, 32P-labeled AluI restriction fragment (residues -56 to 199) containing the entire phospholamban-coding sequence.

DNA Sequence Analysis—Single-stranded DNA templates of the individual subfragments of the genomic DNA cloned into Bluescript vectors were sequenced by the dideoxy method of Sanger et al. (1977), either with the Klenow enzyme and [α-32P]dCTP or with Sequenase (U. S. Biochemical Corp.) and [α-32P]dATP-γ-S. The sequence shown was determined in both strands.

RNA Isolation and Northern Blot Analysis—Total RNA was isolated from rabbit cardiac muscle, slow twitch skeletal muscle (soleus), fast twitch skeletal muscle (plantaris), esophagus, aorta, uterus, bladder, small intestine, large intestine, trachea, brain, spleen, liver, kidney, and testes according to the method of Chomczynski and Sacchi (1987). For Northern blot analysis, total RNA was size-fractionated in 1% agarose gels containing 1 M formaldehyde. The gel was blotted onto nitrocellulose and hybridized with 32P-labeled rabbit phospholamban cDNA probes. The filter was washed under stringent conditions and exposed to x-ray film for 12 h to 6 days.

Primer Extension Analyses—A 31-mer oligodeoxyribonucleotide primer was synthesized that was complementary to the entire phospholamban mRNA. A synthetic 31-mer oligonucleotide, complementary to a part of the first exon (Fig. 2) of the phospholamban gene, was annealed to total RNA from heart (10 μg, lane 1; 100 μg, lane 3) and fast twitch skeletal muscle (10 μg, lane 2; 100 μg, lane 4). The primer-extended products were analyzed by electrophoresis on a 5% sequencing gel. Products 46 and 195–196 bases long can be discerned in the experiments in which heart mRNA was used (lanes 1 and 3), but these are absent in experiments in which fast twitch skeletal muscle mRNA was used (lanes 2 and 4).

Chromosomal Mapping of the Human Phospholamban Gene—The human-rodent somatic cell hybrid series used in this study were those used for the mapping of the fast twitch and slow twitch/cardiac Ca2+-ATPase genes (MacLennan et al., 1987). Ten μg of DNA isolated from parental and hybrid cell lines were digested with EcoRI and transferred to Hybond (Amersham) membrane filters by the method of Southern (1975). After hybridization with the 32P-labeled human phospholamban cDNA probe, the filter was washed under stringent conditions and exposed to Kodak XAR-5 film with an intensifying screen for 3 days.

**Fig. 4.** Primer extension analysis of the 5’ end of the rabbit phospholamban mRNA. A synthetic 31-mer oligonucleotide, complementary to a part of the first exon (Fig. 2) of the phospholamban gene, was annealed to total RNA from heart (10 μg, lane 1; 100 μg, lane 3) and fast twitch skeletal muscle (10 μg, lane 2; 100 μg, lane 4). The primer-extended products were analyzed by electrophoresis on a 5% sequencing gel. Products 46 and 195–196 bases long can be discerned in the experiments in which heart mRNA was used (lanes 1 and 3), but these are absent in experiments in which fast twitch skeletal muscle mRNA was used (lanes 2 and 4).
Northern blot analysis of phospholamban mRNA in various tissues. Seven µg of total RNA from the tissues indicated were separated electrophoretically, blotted onto nitrocellulose, and hybridized with a 377-bp cDNA fragment containing the complete coding region (5' EcoRI linker at position -177 to AluI at position 200) of the rabbit cardiac phospholamban cDNA (Fuji et al., 1988). After washing under high stringency, the filter was exposed to x-ray film for 12 h (A) or 6 days (B).

RESULTS

Isolation of Genomic Clones—Recombinational screening of a 1000-phage from a rabbit genomic DNA library yielded one recombinant phage labeled XrPG1 (Fig. 1). Sequencing of the 5' end of this 17-kb clone showed that it contained the 5'-untranslated sequence of the mRNA transcripts but lacked the coding sequence and the 5'-regulatory sequence of the gene. We were unable to isolate a second clone by recombinational screening using the coding sequence as a probe. We were, however, able to isolate an overlapping recombinant phage clone by hybridization screening of about 4 × 10^6 phages, using as a probe an AluI fragment covering the complete coding sequence of rabbit phospholamban cDNA. In this screen, we isolated a clone containing 6 kb of intron sequence, as well as the second exon containing the complete coding sequence. This clone, shown in Fig. 1, was designated λPG2. As this clone lacked an exon sequence corresponding to the 5'-untranslated mRNA sequence (Fig. 1), rescanning of the library was carried out with an AluI to Rsal fragment (residues -160 to -121 in Fuji et al., 1988) from the 5'-untranslated cDNA sequence as a probe. The third clone, isolated from 1.8 × 10^6 phages and labeled λPG3, contained 8.5 kb of intron sequence, the first exon, and a potential upstream regulatory sequence (Fig. 1).

Structure of the Phospholamban Gene—A comparison of the DNA sequence of the genomic clone (Fig. 1) with the cDNA clone reported previously (Fuji et al., 1988) shows that the rabbit phospholamban gene consists of two exons with one 10.5-kb intron in the middle of its 5'-untranslated sequence (Fig. 2). Southern blot analysis of rabbit genomic DNA isolated from cardiac muscle (not shown) and kidney (Fig. 3), digested with several restriction endonucleases, showed only one band with each digestion. The size of each of the DNA fragments was consistent with its derivation from the same source as the genomic clones isolated here (Fig. 3), indicating that only a single copy of the phospholamban gene exists in the rabbit genome. Since we could not detect any other hybridizing band in the same blot, even under conditions of lower stringency washing (1 × SSCP, 65 °C, not shown), we conclude that no other pseudogenes or homologous phospholamban genes exist. The sequence of the 5'-flanking region, the coding region, and the 3'-flanking region are presented in Fig. 2. The sequences at donor and acceptor sites of the single intron were AAG/gtaag and cag/G, which are consistent with consensus donor and acceptor sequences, respectively (Mount, 1982).

Characterization of the 5'-Flanking Region of the Gene—In order to identify the cap site in phospholamban mRNA, primer extension analysis (Agarwal et al., 1981) using total RNA isolated from rabbit cardiac and fast twitch skeletal muscle (plantaris) was performed (Fig. 4). Two bands arose by primer extension of cardiac muscle mRNA that were not...
present in plantaris mRNA, indicating that these bands were specific for cardiac muscle mRNA. The location of these bands would localize the cap site either 185 or 335 bases upstream from the translation initiation site in mRNA (89 bases from the exon/intron boundary). A canonical CCAAT sequence with the surrounding sequence tattgtaa-CCAATcagaa, similar to the consensus sequence for the site occupied by the human CCAAT-binding protein CP1, CCTNNNNNAA CCAATCAAGT (Chodosh et al., 1988), and a TATA box motif (CATAA) were found upstream of the cap site predicted by the smaller primer-extended product. No such typical sequence elements were found upstream of the larger primer-extended product, however. Moreover, in characterization of more than 10 cDNA clones, we have not observed a 5'-untranslated sequence longer than 185 bases. Thus, the proximal initiator site is likely to be predominant. As most eukaryotic mRNAs begin with a purine residue (Breathnach and Chambon, 1981), the G residue at position −185 is likely to be the predominant transcription initiation site in cardiac muscle.

Phospholamban Gene Expression in Rabbit Smooth Muscle Tissues—Biochemical and immunohistochemical studies suggest that phospholamban is present in some smooth muscle tissues (Raeymaekers and Jones, 1986; Ferguson et al., 1988). Recently, a cDNA clone encoding phospholamban was isolated from a pig stomach smooth muscle library (Verboonen et al., 1989). We also isolated a cDNA clone by screening of 5 × 10^6 phages from the rabbit uterus smooth muscle cDNA library described by Lytton et al. (1989). The restriction map and nucleotide sequence of the coding region were identical with the rabbit cardiac and slow twitch muscle cDNA isolated earlier (not shown).

In order to investigate which tissues express the phospholamban gene, Northern blot analysis of the mRNA from various muscle and nonmuscle tissues was carried out. As shown in Fig. 5, one strong band of about 3.4 kb was found in all muscle tissues. In addition, an upper band and at least one lower band were observed in heart, soleus, and smooth muscle mRNAs. These additional phospholamban mRNAs probably represented polymorphic poly(A) attachment sites (Fujii et al., 1988) or transcription initiation sites. All smooth muscles examined expressed phospholamban mRNA. Low expression was also found in the nonmuscle tissues spleen, testes, and kidney, but their detection required 6 days exposure of the x-ray film. This low level of expression could be explained by smooth muscle contamination from blood vessels in these tissues. Northern blot analysis, together with cDNA cloning, provide a direct demonstration that smooth muscle, as well as cardiac and slow twitch skeletal muscle, express the same phospholamban gene.

Isolation and Characterization of Human Phospholamban cDNA—In a screen of 7 × 10^7 λgt10 recombinant phages from a human muscle cDNA library (Koenig et al., 1987), we isolated one clone using mixed probes from dog (Fujii et al., 1987) and rabbit (Fujii et al., 1988) phospholamban cDNAs. The partial restriction map and the complete nucleotide sequence, together with the deduced amino acid sequence, are shown in Fig. 6. The deduced amino acid sequence of the human cDNA clone differs from both rabbit and dog at position 27 (Lys for Asn) and from dog at position 2 (Glu for Asp) (Fig. 7). Thus, the human phospholamban is more basic than the dog, rabbit, and pig proteins.

Chromosomal Mapping of the Human Phospholamban Gene—The DNA isolated from human-mouse somatic cell hybrids (MacLennan et al., 1987; Willard and Riordan, 1985) was digested with EcoRI and analyzed by Southern blotting, using as a probe a 233-bp PvuII-BglII human cDNA fragment (Fig. 6) that contained the entire phospholamban coding region. Under conditions of high stringency, this probe bound to a single 4.2-kb fragment in human genomic DNA but not in mouse DNA (Fig. 8). The segregation of this 4.2-kb EcoRI band was concordant only with chromosome 6 in this panel of hybrids, and no discordancy was observed for chromosome 6 (Table I).
Phospholamban Gene Structure

DISCUSSION

Phospholamban is one of the smallest membrane proteins known, containing only 52 amino acid residues, and N-terminal acetylation is the only post-translational processing of the initial product (Fujii et al., 1986). The existence of two forms of phospholamban in cardiac sarcoplasmic reticulum was predicted, since phospholamban localized in the terminal cisternae reacted with calmodulin- and cAMP-dependent protein kinases in a different manner from that localized in longitudinal sarcoplasmic reticulum (Gasser et al., 1986, 1988). Nevertheless, we have been able to detect only one sequence by protein sequencing (Fujii et al., 1986) and by cDNA cloning (Fujii et al., 1987, 1988). In this study, we did not detect any pseudogene- or related gene-derived hybridization signals in genomic DNA blots (Fig. 3). This indicates that phospholamban is transcribed from a single gene, that it is composed of only one kind of polypeptide, and that the pentameric form in SDS-polyacrylamide gel electrophoresis (Wegener and Jones, 1984; Fujii et al., 1986) must be a homopentamer. Our expression studies have also supported this conclusion, since the phospholamban expressed in cells transfected with phospholamban cDNA formed a pentamer that could be phosphorylated by both calmodulin- and cAMP-dependent protein kinases (Fujii et al., 1989). Therefore, any changes in reactivity following exposure to different kinases (Gasser et al., 1986, 1988) could only be due to a post-translational rearrangement of a homogeneous polypeptide encoded by a single gene.

Upon cloning and sequencing of the genomic DNA, we found that the phospholamban gene has only one large intron in its 5' untranslated region (Figs. 1 and 2). Although other genes are known to have introns in 5'-untranslated sequences, they also have additional introns in their coding sequences (Breitbart and Nadal-Ginard, 1986; Citri et al., 1987). The sequence CTAAT, 29 bases upstream from the intron-exon boundary, could serve as an internal splicing signal (Keller and Noon, 1984) for this large intron.

We have recently shown that the gene encoding the slow twitch/cardiac muscle Ca^{2+}-ATPase (SERCA2) is expressed in smooth muscle and nonmuscle tissues as an alternatively spliced product (SERCA2b) (Lytton et al. 1989). Analysis of the regulatory region in the genomic clone encoding SERCA2 suggested that the gene has the characteristics of a "housekeeping" gene (Zarain-Herzberg et al. 1990). Phospholamban and cardiac muscle Ca^{2+}-ATPase protein (SERCA2a) expression are known to be triggered at the same stage in sheep cardiac muscle development (Mahony and Jones, 1986), suggesting that expression of phospholamban and the Ca^{2+}-ATPase genes should be developmentally regulated by a similar mechanism. Northern blot analysis (Fig. 5) and cDNA cloning revealed phospholamban mRNA transcripts in cardiac and slow twitch skeletal muscles, in various smooth muscle tissues, and in the nonmuscle tissues, spleen, testes, and kidney. Since the hybridizing bands in the nonmuscle tissues were very faint, even after very long exposure to x-ray film, the mRNA detected in these tissues might be explained as contamination from smooth muscle in blood vessels. Analysis of the 5' region flanking the 5' untranslated sequence revealed no Sp1-binding sequences, suggesting that the phospholamban gene may not be a "housekeeping" gene and that the expression of SERCA2 and phospholamban genes may not be regulated by the same mechanism. In experimentally induced hypertrophic hearts, the expression of phospholamban and the Ca^{2+}-ATPase were not coordinated (Nagai et al., 1989). Since phospholamban is an inhibitory protein in the unphosphorylated state (Tada et al., 1988), any nonmuscle
Ca\textsuperscript{2+}-ATPase existing in the absence of phospholamban should bind Ca\textsuperscript{2+} with a higher affinity compared with cardiac/slow twitch muscles.

The 5′-flanking sequence of the phospholamban gene is AT-rich (about 60%). We found a canonical CCAAT sequence at the consensus position −93 bases from the tentative transcription initiation site and a possible TATA box (CATAA) at position −10. These sequences would be necessary for basal expression of the gene by binding transcription factors. We also found evidence for another possible transcription initiation site, 150 bases upstream from the first site, as seen in Fig. 4. Functional analysis of the promoter will be necessary to confirm the actual promoter sequence.

In earlier studies (Fujii et al., 1988), we found that the long 3′-untranslated sequence of phospholamban mRNA was highly conserved between dog and rabbit and that three different poly(A) attachment sites were utilized. In some mRNA transcripts, the 3′-untranslated sequences are believed to be responsible for efficient cleavage at the poly(A) attachment site (Gil and Proudfoot, 1987). We compared over 100 bases of the sequences, both downstream and upstream of three different poly(A) attachment sites (Fig. 9). These sequences are similar in that they consist of AT clusters intervening with cytidine or guanine residues. Such clusters may participate in the formation of intramolecular loops or double-stranded stalks that could function as transcription termination signals. Alternatively, the transcription machinery might misread these similar sequences appearing before the preferred transcription-termination signal even though the similar sequence elements do not correspond to the consensus sequence reported in the literature (Berget, 1984; Birnstiel et al., 1985; McLauchlan et al., 1985).

The human phospholamban gene has been localized to chromosome 6. Thus, the gene encoding the regulatory protein for the cardiac Ca\textsuperscript{2+}-ATPase is not linked in tandem with the slow twitch/cardiac Ca\textsuperscript{2+}-ATPase gene located on human chromosome 12 (MacLennan et al., 1987) or with any other sarcoplasmic reticulum protein gene localized to date.

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