Single-stranded DNA-binding Proteins PURα and PURβ Bind to a Purine-rich Negative Regulatory Element of the α-Myosin Heavy Chain Gene and Control Transcriptional and Translational Regulation of the Gene Expression

IMPLICATIONS IN THE REPRESSION OF α-MYOSIN HEAVY CHAIN DURING HEART FAILURE*

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The α-myosin heavy chain is a principal molecule of the thick filament of the sarcomere, expressed primarily in cardiac myocytes. The mechanism for its cardiac-restricted expression is not yet fully understood. We previously identified a purine-rich negative regulatory (PNR) element in the first intron of the gene, which is essential for its cardiac-specific expression (Gupta, M., Zak, R., Libermann, T. A., and Gupta, M. P. (1998) Mol. Cell. Biol. 18, 7243–7258). In this study we cloned and characterized muscle and non-muscle factors that bind to this element. We show that two single-stranded DNA-binding proteins of the PUR family, PURα and PURβ, which are derived from cardiac myocytes, bind to the plus strand of the PNR element. In functional assays, PURα and PURβ repressed α-myosin heavy chain (α-MHC) gene expression in the presence of upstream regulatory sequences of the gene. However, from HeLa cells an Ets family of protein, Ets-related protein (ERP), binds to double-stranded PNR element. The ERP-PNR complex inhibited the activity of the basal transcription complex from homologous as well as heterologous promoters in a PNR position-independent manner, suggesting that ERP acts as a silencer of α-MHC gene expression in non-muscle cells. We also show that PUR proteins are capable of binding to α-MHC mRNA and attenuate its translational efficiency. Furthermore, we show robust expression of PUR proteins in failing hearts where α-MHC mRNA levels are suppressed. Together, these results reveal that (i) PUR proteins participate in transcriptional as well as translational regulation of α-MHC expression in cardiac myocytes and (ii) ERP may be involved in cardiac-restricted expression of the α-MHC gene by preventing its expression in non-muscle cells.

Myosin is a chemical-mechanical transducer of motion that acts by converting energy from hydrolysis of ATP into the sliding of myofilaments. The myosin molecule of striated muscles is composed of two pairs of non-identical light chains and two myosin heavy chains (MHC).1 Two expressed MHCs in the mammalian heart are α-MHC and β-MHC (1). These two MHC isoforms are differentially regulated, and this has considerable physiological relevance to myocardial contraction (2). The α-MHC promotes faster shortening velocity of cardiac myofibers because of its high ATPase activity, and hearts with predominant α-MHC contents have lesser ventricular systolic resistance and greater intrinsic capacity to generate blood flow (3, 4). The β-MHC, which has low ATPase activity, leads to greater economy of force generation, and hearts with higher β-MHC content have greater intrinsic ability to generate force (2–4). The relative distribution of the two MHC isoforms is developmentally regulated and varies considerably from species to species. In rodents, β-MHC is the predominant isoform in the embryonic heart, whereas α-MHC is the predominant isoform in the adult heart. As the animal ages, levels of α-MHC become suppressed again (for review, see Ref. 1). Unlike in rodents, ~35% α-MHC mRNA and ~10% protein content are expressed in the adult human heart, and this has been found to be reduced to undetectable levels in failing hearts (5, 6).

The expression of the two cardiac MHC isoforms can be modified by various pathophysiological stimuli such as thyroid hormone, exercise, adrenergic stimulation, cell-contractile activity, and hemodynamic overload. During pressure overload hypertrophy, β-MHC expression is increased with or without reciprocal decrease of α-MHC (7, 8). However, during heart failure, regardless of etiology, α-MHC is invariably reduced (5, 6, 8). In humans as well as in animals, considerable loss of α-MHC content has been implicated to be responsible for the reduced myocardial contractility during heart failure (5, 6, 8). Direct evidence for a causal link between loss of α-MHC and development of heart failure came from experiments in which α-MHC gene was ablated. Although homozygous α-MHC−/− null mice die in utero of gross heart defects, the heterozygous mice (α-MHC+/−) survived and had cardiac myopathies characterized by hypertrophy and interstitial fibrosis (9). These

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1 The abbreviations used are: α-MHC, α-myosin heavy chain; PNR, purine-rich negative regulatory element; ERP, Ets-related protein; CMV, cytomegalovirus; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco’s modified Eagle’s medium; UTR, untranslated region.
hearts had abnormal Frank-Starling pressure/force (length/tension) relations, suggesting propensity toward cardiac failure (9). Similar conclusions were also drawn from studies in which a mis-sense mutation was created in the cardiac α-MHC gene (10). Data showing correlation between declines of MHCs content and reduced intrinsic contractile characteristics of the myocardium also support the idea that a critical level of α-MHC content is essential for normal cardiac pump function (3, 4). However, the mechanism behind the repression of α-MHC during heart failure still remains unknown.

Much information has been accumulated about transcriptional control of α-MHC gene expression. Several positive regulatory elements and their binding factors have been identified such as GATA-4, TEF-1, MEF-2, SRF, and TR (thyroid receptors), which control high level expressions of α-MHC gene in cardiac cell background (11–14). However, mutation of any of these elements does not eliminate gene cardiac specificity, suggesting that they alone are not capable of controlling cell-restricted expression of the α-MHC gene. Furthermore, several recent reports show discordance between the expression level of α-MHC mRNA and protein (15–17). In several models of cardiac hypertrophy, α-MHC content has been seen to change independently of changes in the expression level of α-MHC mRNA, suggesting that a post-transcriptional mechanism controls as well the expression of MHC in response to hemodynamic overload (6, 15–17). However, virtually no information is available thus far concerning post-transcriptional regulation of α-MHC gene expression.

We recently identified a highly conserved purine-rich negative regulatory (PNR) element in the first intronic region of the α-MHC gene (18). This element has some unique characteristics not found in other elements identified so far for α-MHC gene regulation. For example, mutation of the PNR element eliminated α-MHC gene cardiac specificity, resulting in its expression in non-muscle cells where it is normally totally inactive. The repression of α-MHC in cardiac hypertrophy/heart failure was no more apparent when the PNR element was removed, suggesting a role of PNR element in down-regulation of α-MHC gene expression during hypertrophy/heart failure as well (18). These results were also confirmed by others utilizing α-MHC promoter as a tool to design a cardiac-specific adeno-viral expression vector (19). In this paper, we report cloning and characterization of factors binding to the α-MHC gene PNR element. We found that from cardiac nuclear extract two single-stranded DNA-binding proteins analogous to murine PURα and PURβ (for review, see Ref. 20) bind to the plus strand of the PNR element but not to the minus strand, and they negatively regulate α-MHC gene transcription in cardiac myocytes. In addition, an Ets-related protein, ERP (22), derived from non-muscle cell extract, binds to the double-stranded PNR element and inhibits α-MHC expression to almost non-detectable levels. We also present evidence that the single-stranded DNA-binding proteins, PURα/β, participate in transcriptional as well as translational regulation of α-MHC gene expression. Furthermore, we show robust expression of PUR proteins in the myocardium of animals and humans with cardiac failure, again underscoring their role in down-regulation of α-MHC expression in this situation. These data, thus, demonstrate a complex interaction of single- and double-stranded DNA binding proteins to the PNR element that serves to control cardiac-specific expression of the α-MHC gene.

MATERIALS AND METHODS

Plasmid Construction—The pX2 luciferase (ATCC) vector was utilized to generate reporter plasmids. Promoter fragments of the α-MHC (~300/+100 and ~74/–100) and Egr-1 genes (~56/–174) were amplified by PCR and subcloned immediately upstream of the luciferase reporter gene between the HindIII and KpnI sites of the pX2 vector. PURα and ERP expression vectors were generated by subcloning EcoRI/KpnI fragments of genes obtained from the pBS library screening into the EcoRI and KpnI sites of a cytomegalovirus expression plasmid, pCD8. The PURα expression vector (pCDNA3.1-PURα) was provided by Dr. K. Khalil (Temple University, Philadelphia, PA). To generate the pCAT-mRP reporter plasmid, a mRP-CAT fusion cDNA was first synthesized by PCR using the CAT gene as the template and a composite forward primer, comprising a HindIII site and the mRP and the beginning sequences of the CAT gene (AAGCTTGTGCTCCCCGGAGGGG-GAAGGCTCGGCCGACCATG), and the reverse primer, complementary to the end of the CAT gene, including the stop codon and a Xbal site (GACTCTAGATACGCCCCCCG). The PCR product was digested with HindIII and XbaI and subcloned into HindIII and XbaI sites of the pCATlll promoter vector, in the case of the original CAT expression vector (Promega, Inc., Madison, WI). Each construct was confirmed by dyeoxx sequence analysis.

Cell Culture and Transfection—Primary cultures of cardiac myocytes were prepared from 2-day-old neonatal rats as described before (28). After differential plating to eliminate fibroblasts, myocytes were further purified using a Percoll density gradient (Amersham Biosciences) and plated at a density of 4 × 105 in 100-mm plates precoated with 2% gelatin. Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. Non-muscle cells were grown in growth medium containing DMEM supplemented with 10% fetal bovine serum. SoB8 muscle cells were grown in DMEM supplemented with 20% fetal bovine serum, and myocyte cell growth was induced by exposure of confluent cultures to differentiation medium containing DMEM plus 10% horse serum. All cultures contained penicillin (100 units/ml) and streptomycin (100 mg/ml) and were maintained in atmosphere of 5% CO2 at 37 °C.

Cardiac myocytes were transfected 48 h after plating using Tfx reagent (Promega) according to manufacturer’s protocol. SoB8 and HeLa cells were transfected using LipofectAMINE reagent (Invitrogen). The pCMV-b-gal was used as a reference plasmid in all transfections. After 48 h, transfected cells were harvested, and cell lysates were prepared and assayed for luciferase (luciferase assay system, Promega). The pCAT-β-gal was used as a reference plasmid in all transfections. After 48 h, transfected cells were harvested, and cell lysates were prepared and assayed for luciferase (luciferase assay system, Promega), β-galactosidase activities, and protein content (Bio-Rad protein reagent). CAT assays were measured using a CAT-enzyme-linked immunosorbent assay kit according to the protocol given by the manufacturer (Roche Applied Science).

Preparation of Nuclear Extract and the Mobility Gel-Shift Assay—Nuclear extracts were prepared from primary cultures of purified cardiac myocytes or confluent HeLa cells according to the procedure as described previously (14). For the mobility gel-shift assay, single-stranded probes were prepared by 5'-end-labeling of single-stranded oligonucleotides with T4 polynucleotide kinase (Invitrogen) and [γ-32P]ATP. Double-stranded probes were prepared by annealing the single-stranded probe with an excess of unlabeled complementary strand oligo. All DNA probes were purified by gel electrophoresis. The single-stranded probe binding reaction was carried out in a 200 μl containing ~20,000 cpm (0.1–0.5 ng) of the labeled DNA probe, 2–5 μg of the nuclear extract, and 1 μg of poly(dI-dC) as nonspecific competitor. The binding buffer consisted of 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.3 mM MgCl2, 8% glycerol, and 0.5 mM phenylmethylsulfonyl fluoride. After incubation at room temperature for 20 min, the reaction mixtures were loaded on 5% native polyacrylamide gels, and electrophoresis was carried out at 150 V in 0.5 × Tris-buffered EDTA buffer in a cold room. For competition and super-shift assays, unlabeled competitor oligonucleotides or the antibody was preincubated with nuclear proteins at room temperature for 30 min in the reaction buffer before addition of the probe. The DNA oligonucleotides were synthesized at the University of Chicago, Genomic core facility, and RNA oligonucleotides were commercially obtained from Dharmacon, Inc., Lafayette, CO. Single-strand sequences of oligonucleotides used in the study are: PNR (+), 5'-ACCTAGAGGAAAGTGTCTTCTGGAAGTGGGCT; PNR (-) mt, 5'-ACCTAGAGGAAAGTGTCTTCTGGAAGTGGGCT; PNR (+)-32P, 5'-AGGCCCACTTCCACATCTGATTCCTGTTCTAGTATTAGGACATCT; PNR (+)-32P, 5'-AGGCCCACTTCCACATCTGATTCCTGTTCTAGTATTAGGACATCT; P.Ets, 5'-TGCGAGGAGGACCCATTCTTGG; mRP-RNA, 5'-GGUUGCCGGGUAAGGGGGCAAGGUC; mRP-RNA-mt, 5'-GGUUGCCGGGUAAGGGGGCAAGGUC; mRP-RNA-mt, 5'-GGUUGCCGGGUAAGGGGGCAAGGUC; mRP-RNA, 5'-GTGTCGAGGAGGAGGACCCATTCTTGG; Y-box, 5'-CTGAGCGAGGAGGAGGACCCATTCTTGG. In vitro transcription and translation of cDNA Libraries—Library screening was performed using standard screening procedures as described elsewhere (48). A multimter oligo containing four concatenated copies of the PNR element was synthesized. This oligo was labeled by random priming and used as a probe to screen a λ-UniZap fetal rat heart or HeLa cell
Northern analysis of the rabbit RNA were prepared using the RNA isolation kit of Ambion, Inc. For isolation of the element was found to be essential for cardiac-specific expression of the α-MHC gene. This element was normally totally inactive (Fig. 1A). In mobility gel-shift analyses, the double-strand PNR oligo produced two different mobility complexes from muscle and non-muscle cell nuclear extracts, a doublet of high mobility complex with cardiac extract, and two slow mobility complexes with HeLa cell extracts (Fig. 1B).

Cloning and Characterization of Cardiac Nuclear Proteins Binding to the PNR Element—To identify the cardiac factor(s) that binds to this element, a rat heart cDNA library was screened with an oligo probe containing multimers of the PNR element. After several rounds of screening, two positive clones were selected; they were then hybridized with the labeled probe in the presence of either unlabeled wild-type or mutated oligos. For both clones, the excess of unlabeled wild-type PNR oligo. For both clones, the excess of unlabeled wild-type oligos was normally totally inactive (Fig. 1A). In mobility gel-shift analyses, the double-strand PNR oligo produced two different mobility complexes from muscle and non-muscle cell nuclear extracts, a doublet of high mobility complex with cardiac extract, and two slow mobility complexes with HeLa cell extracts (Fig. 1B).

RESULTS

We have previously identified a highly conserved PNR element in the first intronic region of the α-MHC gene. This element was found to be essential for cardiac-specific expression of the α-MHC gene. Mutation of the PNR element eliminated cardiac-specificity of the gene, resulting in high levels of expression of α-MHC gene in non-muscle cells (HeLa), where it was normally totally inactive (Fig. 1A). In mobility gel-shift analyses, the double-strand PNR oligo produced two different mobility complexes from muscle and non-muscle cell nuclear extracts, a doublet of high mobility complex with cardiac extract, and two slow mobility complexes with HeLa cell extracts (Fig. 1B).

Characterization of Non-muscle Cell Nuclear Proteins Binding to the PNR Element—To characterize non-muscle factor(s) binding to PNR, a series of gel-shift analyses were performed using HeLa cell nuclear extract and single- or double-stranded PNR oligos as probes. As shown in Fig. 3A, the PNR plus strand produced four different complexes with HeLa cell extract (C1, C2, NS-1, and NS-2), whereas only two complexes (NS-1 and NS-2) were generated by the minus strand (lane 2). Because NS-1 and NS-2 complexes were produced by both the plus and the minus strand of the PNR, we considered them to be non-specific. We then used double-stranded PNR oligo as a probe, wherein both the plus and the minus strands were labeled. This probe generated two additional complexes, H1 and H2 (Fig. 3A, lane 3). Additionally, when the PNR minus strand was labeled and annealed with an excess of the cold plus strand, the resulting probe produced only the H1 and H2 complexes and no C1 or C2 complexes (Fig. 3A, lane 4). From these results, we concluded that C1 and C2 complexes were generated by the PNR plus strand, whereas H1 and H2 complexes were formed by the PNR double-strand oligo.

To further characterize these complexes, a series of gel-shift competition assays were carried out using oligonucleotides with different protein binding sites. As shown in Fig. 3B, a double-strand PNR oligo with only the plus strand labeled produced four specific complexes (C1, C2, H1, and H2) with the HeLa cell nuclear extract. An excess of oligos corresponding to SRE (CAG-G) with an adjacent Ets site (lane 4) or Ets protein binding site of stromelysin (S.Ets, lanes 5 and 6) and Polyomavirus genes (P.Ets, lanes 11 and 12) competed successfully for the H1 and H2 complex formation, suggesting that an Ets family of protein is involved in forming these two complexes. We also used Y-box oligo as a competitor, which has been previously shown to bind to PUR class of proteins (26).
oligo partially inhibited C1 and C2 complex formation (lanes 7 and 8). As expected, the addition of the cold PNR plus strand in the binding reaction completely inhibited C1 and C2 complex formation (lanes 9 and 10). However, to our surprise we found that the cold PNR minus strand also inhibited C1 and C2 complex formation (lanes 13 and 14). We believe that this is likely due to formation of a double-strand probe in the binding reaction by annealing of the cold minus strand with the labeled plus strand, which are not recognized by the PUR proteins. These results together indicated that H1 and H2 complexes are generated by the double-stranded PNR oligo involving an Ets family of protein and that C1 and C2 complexes are produced by binding of PUR proteins to the plus strand of the PNR element. When the formation of H1 and H2 complexes was inhibited by the competitors (lanes 4, 11, and 12), we noted the faster migrating complexes, C1 and C2, became more intense, suggesting that proteins generating H1 and H2 complexes somehow interfere with the formation of the C1 and C2 complexes.

To further identify the Ets family of protein binding to PNR we screened a HeLa cell cDNA library using double-stranded PNR oligo as a probe. After multiple rounds of screening, we pulled-out nine specific positives binding to this element. These clones were further screened to test their binding ability to an Ets oligo. Filters were probed with the labeled PNR oligo probe in the absence or presence of a 100 {eq}M \text{ excess of unlabeled wild-type (wt) or mutated (mt) PNR oligos. D, two positive clones identified were subjected to in vitro protein synthesis. The molecular masses of the proteins were determined by SDS-PAGE.}
antibody in the gel-shift analysis specifically super-shifted and abolished H1 and H2 complexes generated by the HeLa cell extract (Fig. 3C). These results, thus, demonstrated that an Ets family of protein, ERP, which binds only to double-stranded DNA, is involved in generating two slow migrating complexes with the HeLa cell extract.

The Role of PURα/β and ERP in Transcription Regulation of α-MHC Gene Expression—The identification of PURα/β and ERP protein binding to the α-MHC PNR element raises the possibility of their participation in the cell-restricted expression of the α-MHC gene. To test this possibility expression plasmids encoding full-length rat PURα, PURβ, and/or ERP were co-transfected in primary cultures of cardiac myocytes or Sol8 cells with an α-MHC promoter/reporter plasmid that contained wild-type or mutated PNR element. Overexpression of PURβ repressed the α-MHC reporter gene activity in a concentration-dependent manner. Whereas PURα showed a biphasic effect, modest activation (2-fold) at lower concentration was followed by repression at higher concentrations of the expression plasmid. We then tested the combined effect of PURα and PURβ on the reporter gene activity. As shown in Fig. 4A, only a profound repression effect could be observed when cells were co-transfected with a fixed amount of PURβ and increasing amounts of PURα expression plasmid. The modest activation effect of PURα was no longer detectable, indicating that the dominant negative effect of PURβ overrode the positive effect of PURα protein. Also, there was no effect of either PUR proteins when the PNR element of the reporter gene was deleted or mutated, indicating that the repression effect of these proteins indeed required an intact PNR element. To determine the mechanism for the repression effect of PUR proteins, we analyzed several progressive deletion mutants of the α-MHC promoter/reporter gene. These showed that the PURα/β proteins repressed the reporter gene activity only when the upstream sequences of the α-MHC gene from position 130 bp were intact (data not shown). However, they failed to change the activity of the minimum α-MHC promoter/reporter construct (Fig. 4B). Similar results were obtained when the PNR element was linked to the Egr1 minimum promoter sequences, indicating that PUR proteins do not directly modify the activity of the basal transcription machinery. Rather, their repression effect resulted from their negative cooperation with upstream binding positive regulatory factors of the α-MHC gene promoter.

We next examined the role of ERP protein in PNR-mediated
**Fig. 3. Mobility gel-shift analysis with HeLa cell nuclear extract.**

**A.** Formation of different complexes with single- and double-stranded probes. *N.E.,* nuclear extract. **B.** Competition assay with cold oligos with binding sites of different proteins. *CArG, c-Fos SRP binding sequences with an adjacent Ets site; S.Ets, stromelysin Ets site; P.Ets, Polyomavirus Ets site. The asterisk indicates the labeled strand of the double-stranded PNR probe. C1, C2, H1, and H2 are four specific complexes generated by different probes. NS1 and NS are nonspecific complexes. 1× and 2× indicate excess molar concentrations of a competitor over the probe. **C.** Anti-ERP antibody (Ab.) abolished and super-shifted (SS) H1 and H2 complexes formed with HeLa cell extract.
α-MHC gene expression. Overexpression of ERP also had a concentration-dependent repression effect on the reporter gene activity (Fig. 5A). To test whether any cooperation existed between ERP and PUR proteins we analyzed the combined effect of these proteins on the α-MHC/reporter gene activity. We found no further change in the reporter gene activity by combining PUR proteins with ERP. Increasing the concentration of PUR proteins also failed to produce significant change in the reporter gene activity beyond the effect of ERP alone. We then tested whether titration of a fixed amount of different expression plasmids with increasing amounts of the reporter plasmid could change the reporter gene activity. As shown in Fig. 5B, the repression effect of PUR proteins was restored by increasing the amount of the reporter plasmid, which again could be cancelled by increasing amounts of ERP expression plasmid (0.1 µg) was included as an internal control to normalize for transfection efficiency. For each reporter plasmid β-galactosidase-normalized luciferase activity (mean ± S.E.) is derived from 4–6 different transfection experiments.

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To explore the mechanism of functional antagonism between ERP and PUR proteins, we tested the hypothesis that these proteins compete for binding to the PNR element. In the gel-shift analysis, the labeled double-strand PNR probe was incubated with in vitro synthesized ERP and PURα/β proteins. As shown in Fig. 6, increasing amounts of ERP produced a concentration-dependent slow mobility complex (lanes 2–4), and as expected, PURα/β proteins produced a faster migrating complex (lane 5). However, when a fixed amount of PURα/β proteins was combined with increasing amounts of ERP in the binding reaction, we observed a concentration-dependent decline in the PUR protein binding to PNR associated with pro-
portionate increase of the ERP binding to the probe. This observation was consistent with our results from the competition assay, where abolition of the slow migrating H1/H18528H2 complex promoted formation of faster migrating C1/H18528C2 complexes (Figs. 3, B and C). These data demonstrate that ERP antagonizes PUR/H9251/H9252 binding to the PNR element.

The Role of PUR Proteins in Post-transcriptional Regulation of the α-MHC Gene Expression—Because PURa/b preferentially bind to single-stranded DNA, we questioned whether they could also bind to α-MHC mRNA transcripts. To test this hypothesis we performed gel-shift competition assays in which total heart poly(A)/H11001 RNA was used as a competitor. Much to our surprise we discovered that cardiac mRNA had the ability to inhibit PNR-complex formation. This observation raised an obvious question about the nature of the cardiac mRNA transcript that competes with the PNR element. To identify this transcript we analyzed the sequences in the coding region of α-MHC mRNA and found a similar purine-rich element (named mRP element) in the third exon of the α-MHC gene (Fig. 7A).

The protein binding ability of this element was then examined by performing a series of mobility gel-shift assays. When a DNA oligo corresponding to the mRP element (mRP-DNA) was used as labeled probe, a complex identical to that which occurs with the PNR plus strand was formed (Fig. 7B, lane 1). This complex was inhibited by PUR plus strand oligo and total cardiac mRNA as well as by an RNA oligo corresponding to the probe sequences (mRP-RNA) but not by the mutated oligo (Fig. 7B, lane 6 and 7 versus lane 8). These results, thus, indicate that α-MHC mRP-DNA and -RNA sequences have the ability to bind to single-stranded DNA-binding protein that also interacts with the PNR motif.

To further characterize protein binding to the mRP-DNA we incubated cardiac nuclear extract with protein-specific antibodies and resolved the complex by gel-shift analysis. As shown in Fig. 7B (lane 10) a strong super-shifted complex was observed with anti-PURβ antibody; however, there was no demonstrable effect of anti-PURα antibody seen in this assay (lane 9). These results strongly suggest that the mRP element of α-MHC mRNA binds a protein immunologically related to PURβ.

To examine the role of the mRP sequence in mRNA translation in living cells, we adopted a previously described strategy whereby translational repression is conferred by the protein binding site located within the 5'-untranslated region (5'-UTR) of the reporter RNA (27). For this experiment, we generated a highly active CAT reporter construct in which the mRP element, wild-type or mutated, was placed at the 5'-UTR position of an artificial promoter/reporter gene. During cloning of mRP at the 5'-UTR position, care was undertaken to avoid introducing donor or acceptor splicing sites between the mRP and the CAT cDNA. This promoter/reporter gene (pCAT-mRP) synthesizes a mRP-CAT fusion RNA transcript, as confirmed by primer extension and Southern blot analysis (data not shown). To examine the role of the mRP sequence in gene expression, the pCAT-mRP construct was transfected into Sol8 muscle cells.
with or without a PURβ expression vector. The expression level of PURβ was determined by Western blot analysis using protein-specific antibodies. In these experiments synthesis of CAT mRNA was determined by a semiquantitative reverse transcription-PCR using forward and reverse primers against mRP and CAT gene sequences. The CAT protein output was measured by immunoaassay using CAT-ELISA kit, according to manufacturer’s protocol (Roche Applied Science). Previous studies show that factors binding to the 5′–UTR region of a transcript both inhibit and activate mRNA translational ability (27). As shown in Fig. 8, the presence of the mRP sequence did not change the transcriptional activity of the promoter/reporter gene (Fig. 8, bottom panel). However, the mRP cassette suppressed the CAT protein output by ~50%, reflecting its effect on mRNA translation (Fig. 8). Overexpression of PURβ showed further repression on the synthesis of the CAT protein. We also analyzed the combined effect of PURα and β proteins in this assay and observed augmented inhibitory effect of PURβ on the CAT synthesis, although we failed to detect binding of PURα to mRP. Together, these results demonstrate that PUR-proteins participate both in transcription and translation regulation of α-MHC gene expression.

Expression Levels of PUR Proteins Are Elevated in Failing Hearts—Previously we have shown that the activity of factors binding to the PNR element is increased in cardiac failure and perhaps in some aspects of its pathogenesis.

Discussion

Significant expression of the α-MHC gene is restricted to cardiac myocytes. In recent years, this characteristic of α-MHC expression has been extensively utilized to generate cardiace-specific vectors for transgenic as well as for adenovirus-mediated studies (19, 29). However, the mechanism of the cardiac cell-restricted expression of α-MHC gene remains poorly understood. We have previously identified a highly conserved PNR element in the first intron of α-MHC gene that controls its cardiac cell-restricted expression. The PNR element was also found to participate in down-regulation of α-MHC gene expression during cardiac hypertrophy/failure (18). Studies described here demonstrate that two single-strand DNA-binding proteins from cardiac nuclear extract, PURα and PURβ, bind to the plus strand of the PNR element, whereas an Ets-related protein, ERP, arising from non-muscle cell extract, binds to double-strand of the PNR. Overexpression of PURα and -β modestly down-regulates α-MHC gene expression in cardiac myocytes. We also show that ERP competes with PUR proteins to bind to the PNR element and acts as a silencer to prevent α-MHC gene expression in non-muscle cells. Furthermore, we present evidence that PUR proteins have the capability to bind to the coding region of α-MHC mRNA transcripts and participate in translation regulation of the gene expression.

The PURα/PURβ proteins are single-strand DNA binding proteins that can bind to DNA as the homodimer or heterodimer (20–25). They participate in cell proliferation, differentiation, and apoptosis as well as cell-specific gene regulation. Cellular levels of PUR proteins fluctuate significantly during the cell cycle, and micro injection of PURα into proliferating NIH3T3 cells during the S phase has been shown to cause cell cycle arrest, thus documenting a critical role of PUR proteins in cell division and growth (30, 31). PUR proteins are also highly expressed in cardiac myocytes, and their level changes in diseased hearts; however, except this study we are not aware of any previous report where a role of these proteins in cardiac muscle gene regulation has been reported. Several lines of evidence in our study strongly demonstrate that from the cardiac nuclear extract, PURα and PURβ bind to the plus strand (coding strand) and not to the minus strand of the α-MHC gene PNR element. For example (i) in the gel-shift analysis, the protein DNA complex could be observed only when the plus strand was labeled, (ii) in the competition and antibody supershift assays the PNR-complex was either abolished and/or super-shifted with polyclonal antibodies against either PURα or PURβ proteins, (iii) by screening of a cardiac cDNA library, both PUR proteins were specifically pulled out by the PNR sequences. Earlier reports have shown a role of PURα/β proteins in smooth muscle-specific expression of the vascular α-actin gene. Kelm and co-workers (32) show that PUR proteins bind to the plus strand of a cryptic M-CAT element of the vascular α-actin gene and cooperate with TEF-1 and another single strand DNA-binding protein, MSY1, which binds to the
complementary pyrimidine-rich strand of the M-CAT element. PURα, PURβ, and MSY-1 physically interact and form a repression complex to regulate the cell-specific expression of vascular α-actin gene. In our analysis, however, we found no evidence of MSY-1 or TEF-1 binding to the PNR element. Instead, we found that the Ets-family of proteins, ERP, derived from non-muscle cell extract, binds to the double-strand PNR element. Neither ERP nor PURα/β proteins are tissue-specific, yet we find no evidence of ERP binding to the PNR element arising from cardiac nuclear extract even when low salt conditions of protein binding were used. On the other hand, PUR proteins derived from HeLa cells could bind to the single-strand PNR element, albeit with lower affinity than ERP, which binds to the double-strand PNR element. A relevant question is why only PUR proteins from cardiac extract and not the ERP pro-
tein bind to PNR? The answer may lie in the distribution pattern of these proteins and their ability to bind to other transcription factors. PUR proteins are ubiquitously expressed, whereas ERP expression is limited to certain cell types. In the heart ERP expression is developmentally regulated. It is abundantly expressed in the vascular elements of both fetal and adult hearts; however, in the fetal cardiac myocytes it is expressed at a low level and is almost undetectable in adult heart myocytes (49). In addition, interaction of these proteins with other factors has been shown to modify their DNA binding abilities. Interaction of the PUR protein with factors such as retinoblastoma protein (Rb), E2F-1, cyclin A, and YB-1 has been shown to attenuate its DNA binding ability (26, 33, 34). Conversely, interaction of PUR with Ca$^{2+}$/calmodulin has been shown to augment its DNA binding ability to the recognition sequence (35). Furthermore, Gallia et al. (36) show that binding of the PUR protein to RNA transcripts enhances its self-association and its ability to bind to the target DNA sequence (36). It is, therefore, likely that the binding of PUR proteins to α-MHC mRNA transcripts and/or with other factors promotes its ability to bind to the PNR element. It is also conceivable that inhibitory complexes generated by ERP in cardiac myocytes preclude its binding to the PNR element. ERP is a strong negative regulatory factor that contains two strong inhibitory domains (44, 50). One of them resembles the basic helix-loop-helix motif and has the ability to bind to other basic helix-loop-helix proteins expressed in cardiac myocytes (50). Recently, binding of a basic helix-loop-helix protein, Id, to an Ets family member was shown to inhibit their DNA binding activity to target DNA sequences (37). It is, thus, conceivable that ERP binds to Id or other cardiac basic helix-loop-helix proteins, diminishing its ability to bind to the α-MHC PNR element. Future studies aimed at defining the specific protein complexes that alter the PNR binding ability of ERP would be helpful in further understanding its role in the cell-restricted expression of the α-MHC gene. Such studies are beyond the scope of the present publication.

**PNR Element Participates in Cardiac-restricted Expression of the α-MHC Gene**—Mutation of the PNR element eliminates cardiac-restricted expression of the α-MHC gene, a finding confirmed by others (18, 19). We do not know any other element in the α-MHC gene that has similar credentials. Although PNR is an activator, the combined effect of PURα/β is repression of α-MHC gene expression. The functional significance of the PURα homodimer complex may differ substantially from the homodimer of PURβ or from the PURα/β heterodimer.
Because the doublet generated by cardiac proteins was abolished and super-shifted in this study by polyclonal antibodies against either PUR protein, the majority of the complex was formed by the PURα/β heterodimer, which exerted a negative effect on α-MHC gene expression. Data presented here and reported before demonstrate that the PNR element is functionally active in cardiac myocytes only when the upstream regulatory sequences of the α-MHC gene are present and not within the minimum promoter region, indicating that PUR proteins cooperate with other cardiac factors binding to the α-MHC gene promoter in a negative manner (18). It is of interest to note that PUR proteins have been shown to bind to other cardiac myogenic factors, e.g. TEF-1 and SRF, which also play prominent roles in α-MHC gene expression (14, 32). Recently, YY1 has been shown to bind to an upstream element in the human α-MHC gene promoter where it negatively regulates the gene expression (51). In neurons, YY1 together with PURα controls the cell-specific expression of FE65 gene (24). Thus, PUR proteins are likely to control α-MHC gene expression by regulating the activity of other cardiac factors binding to upstream regulatory region of the promoter.

How does the PNR element prevent α-MHC gene expression in non-muscle cells? ERP is a strong negative regulatory factor and competes with PURα/β proteins to bind to the PNR. Overexpression of ERP antagonized the effect of PUR proteins in our transfection assays, suggesting that ERP inhibited α-MHC expression in part by preventing the binding of PUR proteins to the PNR element. There are many examples of negatively acting factors controlling cell-specific gene expression. Studies on several cell types have demonstrated two main modes of transcription repression, passive and active modes, which control cell-specific gene expression by negatively acting factors (38). In passive repression, the repressors protein down-regulates the activity of one or more transactivating factors by either competing for their DNA binding site or interacting with the positive activator, thereby reducing their transcriptional activation potential. An example is the suppression of interferon-β gene transcription by interferon regulatory factor-2 (IFR-2) whereby IRF-2 competes with an activator protein (IRF-1) for a given DNA binding site (39). In our case, this mode of gene repression does not seem to apply since the PUR protein complex by itself acts as a repression complex. To the contrary, in active repression (also referred as “silencing”), a silencer element, just as the enhancers, can act upon a promoter at a greater distance and independently of its orientation. The main characteristic of active repression is that it can turn off the expression of a gene efficiently irrespective of the nature and number of the activator proteins present in the cell (40). The active mode of repression has been shown to be especially important in cell type-specific regulation of genes, such as neural-specific type II sodium channel and SCG10 gene (41) and erythroid-specific expression of the 15-lipoxygenase and avian cardiac MLC-2 gene (42, 43). Our data show that ERP is capable of inhibiting expression from the minimum α-MHC gene promoter and acts independently of the position of the PNR element in the gene. From these observations, we suspect that PNR functions as a silencer element to direct cell-restricted expression of the α-MHC gene. ERP binding to this element could prevent α-MHC gene expression through different mechanisms (a) by interfering with the basal transcription machinery, leading to inhibition of basal gene expression, (b) by excluding binding of other cardiac regulatory proteins, e.g. PUR proteins, which could lead to disruption of the cardiac myogenic complex, resulting in gene inhibition, (c) by ERP binding to another co-repressor, CtBP, which interacts with histone deacetylases. Criqui-Filipe et al. (44) have recently shown that the inhibitory effect of ERP can be reduced by treatment of cells with a histone deacetylase inhibitor, TSA, raising the possibility for a role of ERP in repressing gene transcription at the level of chromatin. This may be another possibility whereby ERP could inhibit α-MHC gene expression by preventing loosening of chromatin structure in non-muscle cells, leading to gene silencing.

From the foregoing discussion it appears that PURα/β binding to the PNR is part of the bigger cardiac myogenic complex that appropriately regulates α-MHC gene expression in cardiac myocytes. This is identical to the role of PUR proteins in cell-specific regulation of vascular α-actin gene expression. A possible model demonstrating how cardiac cell-restricted expression of α-MHC gene is controlled by PURα, PURβ, and ERP proteins is shown in Fig. 10.

**PUR Proteins Participate in Translational Regulation of the α-MHC Gene**—Because PURα and PURβ bind only to single-stranded DNA, we tested whether they also bind to the α-MHC mRNA transcript. Data obtained from gel-shift competition, super-shift assays, and the protein binding ability of the corresponding DNA and RNA elements strongly indicated that PURβ has the ability to bind to α-MHC mRNA. Furthermore, when DNA sequences corresponding to mRP were attached into the 5′-UTR position of a CAT reporter gene, they appre-
however, PNR is occupied by a heterodimer of PUR/H9251 proteins, but silences translation of the mRNA transcripts (47).

Xenopus erythroid epithelial cells (45). Similarly, MSY1 has been proposed to exclusively recognize the upper DNA strand and represses gene transcription through its negative cooperation with up-stream binding factors such as SRF. The α-MHC mRNA transcripts also recognize PUR proteins, where they attenuate mRNA translational efficiency. The mRNA-dependent sequestration of PUR proteins could, thus, develop a feedback loop to coordinate between transcriptional and translational regulation of α-MHC gene expression.

Reduced the efficiency of CAT protein translation in a manner that was dependent upon nucleotides and PUR protein binding. Although these results do not directly imply a role of PUR proteins when MR sequences are present in another context, they do, however, indicate that PUR proteins have the ability to influence α-MHC gene regulation at the level of translation. It is also conceivable that binding of PUR proteins to newly synthesized mRNA transcript sequesters this protein from the nucleus, leading to de-repression of transcription through a positive feedback mechanism. There are other examples where single-strand DNA-binding proteins have been implicated in the control of both transcription and translation mechanisms of gene regulation. In the case of β-casein gene expression, mRNA-dependent sequestration of a single-strand DNA-binding repressor has been suggested as a mechanism in the hormone-induced expression of the β-casein gene in mammary epithelial cells (45). Similarly, MSY1 has been proposed to have dual roles in germ cell-specific gene transcription in the testes and translation repression of the resulting mRNA (46). An analogous role for FRGY2 protein has been reported in Xenopus, where it augments oocyte-specific gene transcription but silences translation of the mRNA transcripts (47).

In summary, we have identified two single-strand DNA binding proteins, PURα and PURβ, and a double-strand DNA binding protein, ERP, which bind to the α-MHC PNR element. These proteins bind to PNR in a cell-specific manner and appear to participate in cardiac myocyte-restricted expression of the α-MHC gene. Although one should not overstate the importance of a particular group of factors where combinatorial interaction of many factors is likely to control cardiac-specific gene expression, the PUR proteins identified here appear to have some unique characteristics than other factors identified so far with regard to α-MHC gene regulation. They are involved in both transcription and translation regulation of the α-MHC gene expression and, hence, are capable of functioning as part of a feedback loop that controls both processes. PUR proteins form a repressor complex and their cellular levels are markedly increased in failing hearts, suggesting that under certain conditions they may be responsible for repression of α-MHC levels in a decompensated heart. A relevant issue for future studies is whether changing the activity of PUR factors in the heart would significantly alter the pathogenesis of heart failure.

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