Molecular Cloning of Gene Sequences From Rat Heart Rapidly Responsive to Pressure Overload

Issei Komuro, Yoshikazu Shibazaki, Masahiko Kurabayashi, Fumimaro Takaku, and Yoshio Yazaki

Although pressure overload induces cardiac hypertrophy and reexpression of contractile protein isogenes, the molecular mechanism of both is not well understood. We demonstrated the early change in the translational activity of specific cardiac messenger RNA by two-dimensional gel electrophoresis of in vitro translational products. A total of over 400 translational products were detected by fluorography, and the relative predominance of eight species was increased by pressure overload whereas that of two translational products was decreased. We cloned four pressure-overload–responsive complementary DNA clones, pPO-1, -4, -5, and -6, by differential dot blot hybridization. Two clones were increased from the early period after the imposition of pressure overload; the other two were decreased. The expression pattern of each complementary DNA clone in the pressure-overloaded hearts was similar to that in fetal hearts. Pressure overload also induced the additional messenger RNA, which hybridized with pPO-4. This mRNA was also recognized in fetal, neonatal, and adult spontaneously hypertensive rat hearts. The induction of this transcript by pressure overload was not suppressed but, rather, stimulated by cycloheximide. These results suggest that there are some genes that rapidly respond to pressure overload and that may play some role in the development of cardiac hypertrophy. (Circulation Research 1990;66:979–985)

Hemodynamic overload is known to induce cardiac hypertrophy. Since cardiac myocytes lose their ability for replication soon after birth, cardiac hypertrophy results from enlargement of individual cardiac myocytes.1 Cardiac hypertrophy is accompanied by increase in protein synthesis, total RNA content, and messenger RNAs (mRNAs) for various contractile proteins.2–4 Recently, many studies5–8 have demonstrated that qualitative, as well as quantitative, changes in the composition of contractile proteins occur in cardiac hypertrophy. For instance, β-myosin heavy chain and α-skeletal actin mRNAs are reexpressed by pressure overload (PO).6,7 However, the molecular mechanism of how PO induces cardiac hypertrophy and isoform switching remains unknown.

To extend our knowledge regarding the initial action of PO on hearts, we sought early PO-responsive cardiac gene products. We first examined the existence of the early PO-responsive proteins by two-dimensional gel electrophoresis of in vitro translation products. Among the over 400 translational products, changes were observed in 10 of these products as early as 2 hours after PO induction by aortic constriction. We then isolated four complementary DNA (cDNA) clones that are complementary to the PO-responsive cardiac mRNA species. One of these clones hybridized with another transcript. It was expressed 8 hours after constriction of abdominal aorta and also recognized in fetal, neonatal, and spontaneously hypertensive rat hearts. Moreover, the induction of this transcript by PO was independent of protein synthesis.

Materials and Methods

Animal Preparation

To produce PO, male Wistar rats, weighing 180–200 g, were anesthetized with diethyl ether, and the upper part of the abdominal aorta was constricted.
with a hemoclip according to Nair et al.9 Sham-operated animals underwent an identical procedure except for placement of the hemoclip. To investigate the developmental changes, hearts of 12-, 15-, and 18-day-old embryos, 5-day-old neonates, and 40- and 200-day-old adults were examined. Hearts of spontaneously hypertensive rats (SHRs) and Wistar-Kyoto rats (WKYs) were examined as the chronic PO model. Cycloheximide was administered intraperitoneally in a dose that inhibited the overall cardiac protein synthesis ([3H]leucine incorporation) by more than 95%, according to the method of Chaudhuri and Lieberman.10

RNA Preparation

Animals were killed by decapitation at different time intervals after the operation, and the hearts were excised. The atria, great vessels, and right ventricular free walls were removed. The left ventricle was opened, rinsed with cold saline, and quickly frozen in liquid nitrogen. Total cellular RNA was extracted from the left ventricles by the lithium urea method.11 To ascertain the cellular origin of the mRNA corresponding to the cloned cDNAs, RNA was extracted from neonatal rat cardiac myocyte-rich fractions (more than 90% of cells were beating) and from non-muscle cell-rich fractions (less than 20% of cells were beating), which were fractionated by the preplating method.12 Poly(A+) RNA was enriched by oligo (dT)-cellulose chromatography.

Cell-Free Translation and Analysis of In Vitro Translation Products

RNA-dependent cell-free translation was performed with rabbit reticulocyte lysate (Amersham, Arlington Heights, Illinois) and [35S]methionine by the method of Dillmann et al.13 The reactions were started by the addition of 1 μg poly(A+) RNA and incubated at 30°C for 60 minutes. Samples containing equal amounts of radioactivity were subjected to two-dimensional gel electrophoresis.14 After electrophoresis, the gels were prepared for fluorography, dried, and exposed to Kodak XAR-5 films at −70°C. We quantified the PO-responsive spots isolated by two-dimensional gel electrophoresis through measurement of radioactivity. Gel fragments were incubated for 24 hours at 20°C in tissue solubilizer and assayed in a liquid scintillation counter (Packard Instrument, Downers Grove, Illinois). The integrated density of product 0, whose predominance was not affected by acute pressure overload or developmental stage, was used to calculate the corrected integrated densities.

Screening of a cDNA Library

A total of 1,500 single plaques of rat heart cDNA library (purchased from Clontech Laboratories, Palo Alto, California) were isolated with sterile toothpicks, grown individually in microtiter wells, and replicated onto nylon membranes. Five micrograms of poly(A+) RNA isolated from sham-operated and 8-hour–PO hearts was labeled by reverse transcriptase. For differential plaque hybridization, one set of filters was hybridized with 32P-labeled probe complementary to mRNA, which was extracted from sham-operated rat hearts, and the other set of filters was hybridized with an equivalent probe derived from 8-hour–PO rat hearts. Hybridization and autoradiography were performed as described previously.15 Phage DNA that gave a differential signal between duplicate membrane was purified according to Maniatis et al.16 EcoRI-excised cDNA inserts were subcloned into a plasmid vector pUC13 and amplified. Sequencing was carried out by the dideoxynucleotide chain-termination method of Sanger et al.17 Sequence data were analyzed with software from Software Development, Tokyo, Japan.

Northern Blot Analysis

Twenty micrograms of the total RNA or 3 μg poly(A+) RNA were size-fractionated by agarose gels and transferred to nylon membranes. cDNA probes were prepared by the random-priming procedure. Prehybridization and hybridization were carried out as described previously.15 The filters were hybridized sequentially with mouse α-actin cDNA probe to show the integrity of RNA samples.18

Results

Analysis of In Vitro Translation Products

Figure 1 shows representative two-dimensional patterns of translational products encoded by cardiac RNA isolated 0, 2, and 8 hours after PO and from 15-day-old fetal hearts. Sham operation did not change the amount of each translational product significantly (Table 1). The predominance of eight translational products (products 1, 3, and 5–10) was significantly increased whereas that of two products (products 2 and 4) was decreased by acute PO. Some of these PO-responsive alterations occurred in spot pairs of similar molecular weights but slightly different isoelectric points. Product 2 disappeared after PO, but product 3, of which the molecular weight was the same and the isoelectric point was different, appeared. Product 4 was gradually decreased by PO, and correspondingly, product 5 was increased. Although there was a great difference in the translational products between adult and fetal hearts, six out of eight translational products, of which the expression levels were rapidly increased by PO, were also recognized in fetal hearts (Figure 1 and Table 1).

Molecular Cloning of PO-Responsive mRNAs

Since the expression levels of several products were proved to change as early as 2 hours after PO, we isolated such mRNAs as cDNA clones. A total of 1,500 single plaques were picked up in microtiter wells, replicated onto nylon membranes, and screened by differential dot blot hybridization for PO-responsive cDNA inserts. Phage DNA, which scored positive in the initial screening, was purified, and Northern blotting was performed to discard false
positives. A total of four PO-responsive clones, designated pPO-1, -4, -5, and -6, survived, and the size of each cloned cDNA was 1.5, 2.2, 2.0, and 2.5 kilobases, respectively. The size of their corresponding mRNAs ranged from about 1.5 to 7.6 kilobases by Northern blot analysis (Figure 2). Their sequence analysis demonstrated that these four cDNA clones showed no homologies with any known sequences including contractile proteins.

**Northern Blot Analysis of PO-Responsive Clones During Acute PO and Expression of These Clones in Cultured Cells**

Figure 3 shows the expression patterns of these clones during acute PO. Sham operation did not change the expression levels of any clones. The expression level of pPO-1 was increased at 4 hours after PO, peaked at 8 hours, and decreased to the basal level at 24 hours. In pPO-4, not only the expression level was increased but also another transcript appeared 8 hours after aortic constriction. The expression level of pPO-5 was decreased gradually,

**TABLE 1. Corrected Integrated Density Values**

<table>
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<tr>
<th>TP</th>
<th>M_\text{r} (kDa)</th>
<th>pI</th>
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<th>2 hr</th>
<th>8 hr</th>
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<th>Fetus</th>
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<tr>
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</table>

Each value is the mean of triplicate determinations. The corrected values for integrated densities represent ratios of integrated densities of identified translational product to that of a nonresponding product. PO, pressure overload; TP, translational product; M_\text{r}, molecular weight ratio; kDa, kilodalton; pI, pH value for the isoelectric point; sham, translational products of 8 hours after sham operation; fetus, translational products from 15-day-old fetus.
and that of pPO-6 was transiently decreased between 8 and 12 hours after the operation. The expression levels of the mRNAs corresponding to these four cDNAs were about four to eight times more abundant in cardiac myocyte-rich fractions than in non-muscle cell-rich fractions.

**Northern Blot Analysis of PO-Responsive Clones During Developmental Stage**

The expression of these PO-responsive clones was examined during physiological growth. As shown in Figure 4, the expression pattern of each PO-responsive clone during fetal and neonatal stages, when cardiocytes are growing by proliferation, was similar to that during PO-induced hypertrophy. The expression level of pPO-1 was high around birth, and two transcripts that hybridized with pPO-4 were also recognized only at fetal and neonatal periods. On the other hand, the expression levels of pPO-5 and -6, which were decreased by PO, were quite low during fetal period.

**Northern Blot Analysis of PO-Responsive Clones During Chronic PO**

To ascertain the expression of these clones during chronic PO, we examined SHR hearts as a chronic PO heart model. The expression levels of pPO-1, -5, and -6 did not significantly change in SHR hearts compared with WKY hearts, but the two mRNAs hybridized with pPO-4 only in SHR hearts. Of interest was that the larger size mRNA was detected from the prehypertensive stage (4 weeks old) (Figure 5).

**Induction of the Two mRNAs Corresponding to pPO-4 by Cycloheximide**

To examine the mechanisms of the induction of the two mRNAs corresponding to pPO-4, we injected a potent protein synthesis inhibitor, cycloheximide, intraperitoneally before aortic banding. Cycloheximide (50 mg/kg body wt) blocked the protein synthesis more than 95% in the heart (data not shown). The induction of the additional mRNA by PO was not suppressed by cycloheximide. On the contrary, it was induced by cycloheximide injection alone (Figure 6).

**Discussion**

We demonstrated the existence of proteins of which the expression levels were rapidly changed by acute PO in the rat heart and isolated four PO-responsive cDNA clones. The expression patterns of these PO-responsive clones in the hypertrophic hearts were similar to those in fetal and neonatal hearts. Although there were no homologies in these cloned cDNAs with known sequences, these four PO-responsive clones were expressed in myocytes of the hearts. One of these PO-responsive clones, pPO-4, hybridized with two distinctive transcripts. The PO-induced additional mRNA corresponding to pPO-4 was also expressed in fetal and neonatal hearts during the developmental stage and in SHR hearts from the prehypertensive stage. The induction of this mRNA was not inhibited but rather induced by protein synthesis inhibitor.

Many data have been accumulated confirming that PO induces not only quantitative changes but also qualitative changes in the heart.\(^5\,\sim\,8\) Fetal isoforms of the contractile proteins were reexpressed by PO. For example, \(\alpha\)- to \(\beta\)-myosin heavy chain transition occurs in rat hearts by PO.\(^5\,\sim\,6\) The question remains as to how PO induces the cardiac hypertrophy and isoform switching. In this acute PO model, the increment of heart weight was detected 2 or 3 days after aortic constriction, and the mRNA of \(\beta\)-myosin heavy chain increased after 24 hours (data not shown). Recently, Boheler and Dillmann\(^19\) showed the alteration of eight translational products in rat hearts after 8 days of aortic constriction. We examined more rapidly responsive proteins, since the possibility might exist that these proteins play an important role in the subsequent events.\(^20\,\sim\,21\)

By in vitro translation study, 10 of 400 individual cardiac mRNA rapidly responded to PO. Some of
their expression levels changed as early as 30 minutes after PO induction (data not shown). Although translatable mRNAs were quite different between adult and fetal hearts, most of PO-responsive mRNAs existed in fetal hearts. There have been many reports that fetal isoforms of contractile proteins are induced by PO. The present study showed that there was a similar expression pattern also in these rapidly PO-responsive proteins between fetal and adult PO hearts. Although cardiocytes lose their ability to replicate after birth, cell hypertrophy of adult myocytes and cell division of fetal myocytes may share a common mechanistic pathway.

**Figure 3.** Northern blot analysis of pressure-overload–responsive clones (pPO-1, -4, -5, and -6) during acute pressure overload and expression of these clones in cultured cells. Aortas of male Wistar rats were constricted with a hemoclip, and rats were killed at indicated times (degrees indicate hours) after operation. RNA was extracted from left ventricles or cardiac cultured cells of neonatal rat. Poly(A+) RNA (3 μg) from left ventricles or 20 μg total RNA from cultured cells was separated on 1.2% agarose gel, stained with ethidium bromide, blotted onto a nylon membrane, and hybridized to pressure-overload–responsive complementary DNA clones. Autoradiograms were exposed for 2 to 5 days with intensifying screen at −70°C. The same RNA blot was sequentially hybridized with mouse α-actin complementary DNA probe as an internal control. In the case of total RNA extracted from cultured cells, parallel samples of stained 28S ribosomal RNA are shown below the autoradiogram. Sham, RNA extracted 8 hours after sham operation; myocytes, RNA extracted from cardiac myocyte–rich fraction; non-myocytes, RNA extracted from non-muscle cell–rich fractions.

**Figure 4.** Northern blot analysis of pressure-overload–responsive clones (pPO-1, -4, -5, and -6) during developmental stage. Hearts of 12-, 15-, and 18-day-old embryos (12d, 15d, and 18d, respectively), 5-day-old neonates (Neo.), and 40- and 200-day-old adults (40d and 200d, respectively) were examined. Procedures of Northern blot analysis were described in Figure 3 legend.

**Figure 5.** Northern blot analysis of pressure-overload–responsive clones (pPO-1, -4, -5, and -6) during chronic pressure overload. Hearts of 4-, 8-, and 12-week-old (4w, 8w, and 12w, respectively) spontaneously hypertensive rats (SHR) or Wistar-Kyoto rats (WKY) were examined. Procedures were the same as described in Figure 3 legend.
To ascertain more about the PO-responsive mRNA, we isolated four cDNA clones by differential hybridization technique. Since the expression levels of PO-responsive proteins and some protooncogenes, c-fos and c-myc, reached the peak 8 hours after aortic constriction,\(^1\) we used the mRNA of sham operation and the time of 8 hours after PO for differential hybridization study. We isolated four PO-responsive clones. Although their expression patterns in PO hearts differed from each other, they were similar to those in fetal hearts. In one clone, pPO-4, another transcript was induced by acute PO and was detected in physiologically growing fetal and neonatal hearts. Moreover, it was abundant in 4-week-old SHR hearts. Since the SHR blood pressure increases from 7 to 9 weeks of age, the expression of this mRNA may be independent of PO. The SHR heart has been reported to show hypertrophy from the prehypertensive state.\(^2\) When these results and observations are taken together, the additional transcript of pPO-4 appears to exist generally in the growing heart. Although the reason the mRNA levels of the other clones did not change in the SHR and the WKY was not clear, the duration and the degree of PO may be related to the expression levels.

The additional transcript that hybridized with pPO-4 was induced by PO even if protein synthesis was inhibited by the prior injection of cycloheximide. This result suggests that the induction of this mRNA that hybridized with pPO-4 might not require other protein synthesis. There might be several possibilities in the expression of the additional transcript: the change in the transcriptional rate or in the mRNA stability, differential processing, or splicing. It is not known from the data presented here. However, since the additional mRNA was induced by cycloheximide injection alone, the expression of this gene may be inhibited by rapidly turning over inhibitory proteins. Although the roles of these clones are unknown, these PO-responsive clones might be useful tools for studying molecular mechanisms of cardiac hypertrophy.

**References**


KEY WORDS • cardiac hypertrophy • cardiac messenger RNA • quantitative fluorography • differential hybridization • Northern blotting
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