Selective Gene Expression in Failing Human Heart

Quantification of Steady-State Levels of Messenger RNA in Endomyocardial Biopsies Using the Polymerase Chain Reaction

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Background. Evaluation of gene expression in failing human heart has been limited by the availability of cardiac tissue.

Methods and Results. We used the polymerase chain reaction (PCR) to assess gene expression in small quantities of failing and nonfailing human heart. PCR is a powerful new molecular biological tool that allows a small quantity of DNA to be amplified as much as 1 million–fold. Total RNA was extracted from 3–5 mg samples of human heart and reverse-transcribed to complementary DNA (cDNA). With selected oligonucleotide primers, we used PCR to amplify cDNAs encoding atrial natriuretic peptide, β-myosin heavy chain, phospholamban, and cytoskeletal β-actin. To quantify the relative levels of messenger RNA (mRNA) in human heart, a known amount of a control RNA was present in the reverse transcription and PCR reactions. The amount of mRNA in the sample could therefore be assessed in relation to the amount of control product. The control RNA was transcribed from a synthetic DNA template containing primers complementary to those used to amplify the cDNAs of interest. Atrial natriuretic factor mRNA could not be detected in nonfailing human heart but was abundant in ventricular myocardium from failing human heart. In contrast, steady-state levels of phospholamban mRNA decreased, whereas levels of β-myosin heavy-chain mRNA were unchanged with heart failure.

Conclusions. Alterations in gene expression in the failing human heart appear to be selective. In addition, the present study suggests that PCR provides a rapid and economical way to quantify the expression of multiple genes of interest in endomyocardial biopsy specimens and may therefore be used to advance our understanding of heart muscle disease. (Circulation 1991;83:1866–1872)

Alterations in steady-state levels of messenger RNA (mRNA) usually parallel changes in the levels of their protein products; therefore, they provide insight into the basis for functional protein modifications. Although these measurements would be useful in advancing our understanding of human heart disease, measurements of mRNA in human tissue have been limited to the large (gram quantities) amounts of myocardium that can be obtained from patients with idiopathic dilated cardiomyopathy who are undergoing cardiac transplantation.1,2 However, recent technological advances have provided the tools necessary to assess gene expression in small quantities of tissue, that is, isolation and purification techniques for use with small quantities of RNA3 and the polymerase chain reaction (PCR) for amplification of small quantities of DNA.4,5

The PCR uses a sequence of three temperature-dependent chemical steps that results in the exponential amplification of a defined segment of DNA by more than 1 million–fold: 1) denaturation of double-stranded DNA at a high temperature, 2) annealing of

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TABLE 1. Oligonucleotide Sequences of 5’ and 3’ Primers for Polymerase Chain Reaction Amplification of Human Cardiac Genes

<table>
<thead>
<tr>
<th>Messenger RNA</th>
<th>5’ Primer</th>
<th>3’ Primer</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANF</td>
<td>5’CAACGCAGACCTGATGGATT3’</td>
<td>3’AGACTAGCTAGACGGAGGATT5’</td>
<td>234</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5’ATCATGAAGTGACGTCGAC3’</td>
<td>3’ACTTCCACTGTCGACGC5’</td>
<td>554</td>
</tr>
<tr>
<td>PHLB</td>
<td>5’TCAATACCTCACTCGCTCTGC3’</td>
<td>3’CTAGACGTAGTAGCAGTAC5’</td>
<td>140</td>
</tr>
<tr>
<td>β-MHC</td>
<td>5’ATCAAGGAGCTACCTACACAG3’</td>
<td>3’GACCTCGGACACATTGTCGA5’</td>
<td>336</td>
</tr>
</tbody>
</table>

ANF, atrial natriuretic factor; β-actin, cytoskeletal β-actin; PHLB, phospholamban; β-MHC, β-myosin heavy chain.

Two convergent DNA primers to opposite strands of the target DNA at low temperatures (40–50°C), and 3) extension of the annealed primer along the DNA strands at 72°C by the heat-stable Taq polymerase in the presence of excess DNA triphosphates. Each set of three steps (or one cycle) results in production of extension products of each primer. These extension products then serve as templates for the second primer in the subsequent cycle, and successive cycles result in an exponential increase in the amount of DNA. The specificity of the PCR reaction is provided by the two oligonucleotide primers that are complementary to base pair sequences that flank the DNA segment to be amplified. When present in excess, these primers compete with the complementary DNA (cDNA) strands to form primer-DNA complexes at low temperatures (40–50°C). PCR was initially used to determine whether small quantities of selected sequences of DNA exist in a given clinical specimen. However, results from a recent study demonstrated that PCR may also be useful in quantifying steady-state levels of mRNA in small quantities of cells or tissue.

In the present study, we used PCR to quantify expression of the genes encoding atrial natriuretic factor (ANF), phospholamban, β-myosin heavy chain, and β-actin in small endomyocardial biopsy-sized samples of human myocardium obtained during cardiac transplantation and endomyocardial biopsy. These mRNAs range from low to high abundance in human heart with ANF mRNA nondetectable in normal adult ventricular myocardium. The demonstration of a substantial and selective expression of ANF mRNA in failing human ventricular myocardium provides a molecular marker of human heart disease. In addition, the ability to measure steady-state levels of cardiac mRNA in endomyocardial biopsies of human heart provides a new approach to understanding the molecular and cellular mechanisms that contribute to myocardial disease.

**Methods**

Left ventricular myocardium from patients with idiopathic dilated cardiomyopathy was obtained at the time of cardiac transplantation as described previously. Nonfailing control hearts were obtained from organ donors whose hearts could not be used for transplantation because of the lack of a suitable recipient or because the donor was more than 50 years old. Endomyocardial biopsies were obtained at the time of pretransplant diagnostic procedures in patients with New York Heart Association functional class III or IV heart failure who were subsequently diagnosed as having idiopathic dilated cardiomyopathy. Both explanted heart tissue and biopsy samples were immediately frozen in liquid nitrogen and stored at −70°C. All tissues were harvested after obtaining informed consent.

Total cellular RNA was isolated from 3–5 mg of frozen ventricular myocardium using a modification of the acid guanidinium thiocyanate/phenol/chloroform extraction. The frozen tissue was homogenized in 1.5 ml of RNAzol B (Cinna/Biotex, Inc., Friendswood, Tex.) with a Polytron (Brinkman Instruments Co., Inc., Westbury, N.Y.), and RNA was stored in ethanol at −70°C. RNA concentration was assessed spectrophotometrically using a Beckman DU-65 (Beckman Instruments, Inc., Fullerton, Calif.) and a Five Carat Cell (Beckman Instruments, Inc.). First-strand cDNA was synthesized from total RNA using reverse transcriptase and oligo-dT primers according to manufacturer’s instructions (Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

Oligonucleotides were synthesized on an Applied Biosystems Inc. DNA synthesizer by The Johns Hopkins University School of Medicine Protein-Peptide Facility. The sequences used for the oligonucleotide primers were selected from regions of the mammalian genes encoding proteins of interest, and, wherever possible, the 5’ and 3’ primers spanned splice junctions (Table 1). Oligonucleotides were end-labeled with [γ-32P]ATP by using T4 polynucleotide kinase (Pharmacia LKB Biotechnology, Piscataway, N.J.).

Synthetic DNA templates were constructed (Figure 1) that contained two pairs of primers complementary to those used to amplify two cDNAs of interest. These synthetic oligonucleotides had sequences for the bacteriophage T7 promoter on their 5’ end for transcription into RNA. The templates also carried polyadenine tracts at their 3’ ends to facilitate reverse transcription by oligo-dT. Control RNA product was separated from control DNA after in vitro transcription using phenol/chloroform extraction and ethanol precipitation after incubation with RNase-free DNase (Promega, Madison, Wis.).

The PCR was carried out in a total volume of 100 µl containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 0.001% (wt/vol) gelatin, 160 µM dNTPs, 13 pmol of each primer, and 1.25 units of *Thermus aquaticus* DNA polymerase (Taq polymerase) (Per-
kin-Elmer/Cetus Corporation, Norwalk, Conn.). A trace amount of $^{32}$P-labeled 3' primer was added to provide $3 \times 10^6$ cpm. The mixture was overlaid with mineral oil and amplified with a TempCycler (Coy Corp., Ann Arbor, Mich.). Each reaction also contained varying quantities of control cDNA prepared by reverse transcription of the in vitro transcription product of the synthetic DNA template. Amplification temperatures were 94°C for 1.5 minutes for denaturation, 40–50°C for 1.5 minutes for primer annealing, and 72°C for 1.5 minutes for primer extension. The primer annealing temperatures varied according to the specific primers being used for amplification.

To ensure that measurements were performed during the exponential phase of amplification, 10 µl of each PCR reaction mixture was removed during successive cycles of amplification (12 to 30) and electrophoresed in a 3% (wt/vol) NuSieve GTG (FMC BioProducts, Rockland, Me.)/0.5% (wt/vol) SeaKem LE (FMC BioProducts) agarose gel containing Tris acetate/EDTA and ethidium bromide. For quantitative analysis, reactions were performed with varying concentrations of control RNA and total RNA to produce a standard curve. A Hae III digest of φ×174 DNA (BRL Life Technologies, Inc., Gaithersburg, Md.) was used as a molecular size standard. Gels were visualized with indirect UV irradiation and photographed, and appropriate bands representing amplification products from the cDNA of interest and amplification products of the control cDNA were cut out from the gel. Radioactivity in the bands of interest was determined by Cerenkov counting. The amount of radioactivity in the excised gel bands was plotted against either the number of PCR cycles or the concentrations of the control RNA.

Products were sequenced to confirm amplification of expected product. After asymmetric amplification (1:50 dilution of 5' primer) was performed, oligonucleotides and excess dNTPs were removed from the amplified DNA by spin dialysis with a Centricon-30 microconcentrator (Amicon, Danvers, Mass.), and the DNA was sequenced by the dideoxy chain termination method (Sequenase 2.0, United States Biochemical Corp., Cleveland, Ohio).

**Results**

The oligonucleotides of 5' and 3' primers of the target genes are shown in Table 1. Each of these pairs of primers produced amplification products from human heart RNA that had been reverse-transcribed into cDNA. That these amplification products represented the appropriate regions of the genes of interest was confirmed by determination of their nucleotide sequences. Because all except the primers for the β-adrenergic receptor and phospholamban (an intronless gene) spanned splice junctions, it is unlikely that these products arose by amplification of contaminating genomic DNA.

Because of tube-to-tube variabilities in amplification efficiency, quantification of target cDNA requires the presence of an internal control. By including a known quantity of an internal control RNA in the reverse-transcription reaction and the resulting cDNA in the amplification reactions, a standard curve can be generated for quantifying the amount of target mRNA in an experimental sample as long as the amplification product of the sample mRNA and the control RNA can be separated by size on an agarose gel. Furthermore, inclusion of the control RNA in the reverse-transcription reaction allows for a control at this initial step in the experimental procedure. We constructed templates having two primer pairs, which produced amplification products of approximately 72 base pairs—substantially different from the product sizes of the target mRNAs (Figure 1).
In Figure 2B, 1 µg total RNA prepared from an endomyocardial biopsy obtained from a patient with idiopathic dilated cardiomyopathy and 1.7×10⁶ molecules of a control template containing the primer sets for ANF amplification were reverse-transcribed in the same reaction mix. An aliquot representing 1/40 of the resulting cDNA mixture was amplified for 12 to 30 cycles in the presence of the ANF primers. At these predetermined ratios of human heart cDNA and control cDNA, the rates of amplification were exponential for both products when the amounts of radioactivity in the two products were plotted as a function of the number of amplification cycles (12 to 30 cycles).

This relation was established independently for each pair of primers and varied according to the efficiency of amplification for each primer pair. Under our experimental conditions, the slopes of the curves for the heart cDNA and the control template cDNA were similar; therefore, the amplification efficiency could be assumed to be the same for both cDNAs within the exponential ranges. This was not unexpected because the same primers are used to amplify the control template as well as the cDNA of interest in the same reaction. As seen in Figure 2A, there was a linear relation between increasing quantities of control RNA and the radioactivity in the excised bands. Furthermore, at the preestablished ratios, the product yields with increasing concentrations of control RNA and total RNA (sample RNA) were colinear over several orders of magnitude. Nonspecific amplification resulted in “tailing” at higher amplification cycles (Figure 3); however, only distinct bands were evident at amplification cycles that were used for quantitative analysis (cycles 18–22).

We obtained the concentration of the sample RNA spectrophotometrically. The use of this technique was verified by constructing a standard curve for RNA samples of known quantity (0.1–2.0 µg) and demonstrating a linear relation with increasing concentrations. The amount of control RNA in the reverse-transcription reaction was adjusted so that the ratios of sample (1 µg) and control cDNA would allow for equal amplification efficiency. However, to ensure that all measurements were within the exponential ranges and that the slopes of the amplification curves for both the control and sample DNA were similar, an amplification curve was performed with each sample. Quantitative analysis was performed using a standard curve constructed with varying concentrations of control RNA (see Figure 2A). The number of amplifications used to construct the standard curve was determined from the amplification curve and was in the exponential (linear) range. Values are expressed as mRNA divided by total RNA.

The yields of total RNA from the endomyocardial biopsy samples of human heart were consistently between 0.6 and 3.0 µg (average yield, 2 µg). One microgram of the total yield is added to the reverse-transcription reaction; the final product provides adequate sample for multiple PCR reactions. This illustrates a major advantage of this technique; considerably more RNA would be required for Northern-blot analysis of multiple mRNAs.

Spectrophotometric measurement of mRNA has some inherent inaccuracy. Furthermore, it is impossible to detect whether failing heart might have a generalized decrease or increase in mRNA synthesis that would account for an increase in the steady-state levels of a single transcript of interest. For these reasons, we normalized all values to the mRNA levels of cytoskeletal β-actin. This mRNA has been used previously for normalization of ANF mRNA.
levels in animal models of heart failure\textsuperscript{12} as well as in other studies of gene expression in animal\textsuperscript{13} and human heart.\textsuperscript{1}

With these techniques, we assessed the relative differences in mRNA levels in endomyocardial biopsy-sized samples from failing and nonfailing human heart and endomyocardial biopsies from patients with idiopathic dilated cardiomyopathy. As seen in Table 2, ANF gene expression could not be appreciated in normal ventricular myocardium. In marked contrast, substantial amounts of ANF mRNA were present in both end-stage failing human heart as well as endomyocardial biopsies from patients with idiopathic dilated cardiomyopathy. It is unlikely that a generalized increase in mRNA synthesis in the failing heart could account for these changes because other mRNAs of interest showed variable regulation in heart failure (Table 2). There was a small decrease in steady-state levels of the mRNA encoding cytoskeletal \(\beta\)-actin in the failing hearts used in the present study compared with nonfailing controls. However, there was a sevenfold decrease in the steady-state levels of mRNA encoding phospholamban, whereas the levels of mRNA encoding \(\beta\)-myosin heavy chain were not altered in the failing human heart. This relation was unaltered by normalizing the levels of phospholamban and \(\beta\)-myosin heavy-chain mRNA to that of \(\beta\)-actin in each sample. There was

**Table 2.** Messenger RNA Levels in Failing and Nonfailing Human Heart

<table>
<thead>
<tr>
<th>Molecules mRNA/(\mu)g total RNA ((\times 10^6))</th>
<th>Failing</th>
<th>Nonfailing</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANF</td>
<td>1.7±0.8 (9)</td>
<td>Not detected (8)</td>
<td>*</td>
</tr>
<tr>
<td>(\beta)-MHC</td>
<td>4.4±1.5 (7)</td>
<td>9.1±2.3 (8)</td>
<td>NS</td>
</tr>
<tr>
<td>Phospholamban</td>
<td>1.1±0.4 (7)</td>
<td>7.5±1.0 (8)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>(\beta)-Actin</td>
<td>0.5±0.09 (7)</td>
<td>1.2±0.1 (8)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Molecules mRNA/molecules (\beta)-actin mRNA</td>
<td>10.9±3.2</td>
<td>8.5±2.2</td>
<td>NS</td>
</tr>
<tr>
<td>Phospholamban</td>
<td>3.1±1.1</td>
<td>6.4±0.9</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

ANF, atrial natriuretic peptide; \(\beta\)-MHC, \(\beta\)-myosin heavy chain; \(\beta\)-actin, cytoskeletal \(\beta\)-actin.

Values are expressed as either per total RNA or per molecule of \(\beta\)-actin messenger RNA (mRNA) in the sample and represent mean±SEM. Values within parentheses represent \(n\).

Endomyocardial biopsy-sized specimens (2–4 mg) were removed from samples obtained from patients with idiopathic dilated cardiomyopathy at the time of cardiac transplantation or from potential organ donors whose hearts could not be used for transplantation. Alternatively, myocardial tissue was also obtained at the time of endomyocardial biopsy in patients subsequently diagnosed as having idiopathic dilated cardiomyopathy (four of nine samples).

*Lowest concentration detectable with standard curve was 1.1×10\(^6\) molecules.
no significant difference between levels of mRNAs of interest in the endomyocardial biopsy specimens compared with samples obtained from hearts acquired at the time of transplantation.

Discussion

It has recently been demonstrated that mRNA levels can be quantified using the PCR and a synthetic RNA as an internal standard.7 In the present study, we demonstrate that by using a modification of these techniques, oligonucleotide primers of designed specificity can amplify small quantities of cDNA transcribed from nanogram quantities of human heart RNA. The presence of a control RNA in the reverse-transcription and amplification reactions provides a method for quantification of multiple mRNAs of interest, which obviates inherent assay-to-assay variations in sample preparation, reverse transcription, and gene amplification. In contrast to the previous report,7 we constructed small synthetic templates containing the T7 promoter at the 5' end and an adenine tract at the 3' end. These small genes are easy to construct and inexpensive because they consist of relatively few base pairs, can be amplified by PCR, and do not require subcloning. Furthermore, the small products (72 base pairs) produced by amplification of these control RNAs can be readily separated from the larger mRNA amplification products.

Theoretically, the efficiencies of both cDNA synthesis and PCR amplification may differ for the sample mRNA and the control RNA because of differences in the distance between the PCR priming sites, lengths of the poly A tails, and nucleotide sequences. However, we have not noted a significant difference in the efficiency of PCR amplification for the mRNAs and control templates in the present study. Furthermore, possible differences in cDNA synthesis should remain the same from sample to sample. It is important to note that in qualitative assessments with a standard control RNA, the concentrations of the control DNA can adversely affect the amplification efficiency of the sample cDNA. Therefore, it is critical that each primer set be evaluated to ensure that the range of concentrations for both the sample cDNA and the control DNA allow amplifications within the exponential range when 10–30 amplification cycles are performed.

A limitation in quantifying mRNAs in small samples of tissue is that it is difficult to determine the denominator because the small sample size precludes accurate assessment of total RNA. However, the recent availability of sensitive spectrophotometric instruments and microcuvettes provided an accurate method with which to determine the quantity of small amounts of RNA. In addition, we also expressed the levels of mRNAs of interest in relation to the quantity of the β-actin mRNA in the same sample. Cytoskeletal actin mRNA has been widely used as a “control” for normalizing mRNA levels in both cardiac and noncardiac tissues.1,12–14 In the group of hearts used in the present study, there was a small but statistically significant decrease in the steady-state levels of β-actin mRNA in the failing human hearts compared with nonfailing controls. However, there was a statistically significant difference in phospholamban mRNA in samples from failing human heart when the data were expressed as either per total RNA in the original sample or a ratio of β-actin mRNA. Similarly, steady-state levels of β-myosin heavy-chain mRNA were not altered by the heart failure state when data were expressed by the two methods. It is unclear whether the decrease in steady-state levels of β-actin mRNA represents an alteration in β-actin gene expression with heart failure or a decrease in mRNA yield from the myopathic tissue; however, our failure to find a difference in steady-state levels of β-myosin heavy-chain mRNA in the two groups would suggest the former. In either case, it is clear from the present results that both ANF and phospholamban gene expression are substantially altered in failing human heart.

It is well known that levels of circulating atrial natriuretic peptide are increased in patients with congestive heart failure15; however, ANF gene expression has not been assessed in the ventricular myocardium of patients with congestive heart failure. Our finding that ANF mRNA is nondetectable in nonfailing adult ventricular myocardium is consistent with a previous report,8 and the substantial increases in ANF mRNA in samples of failing human heart are consistent with data from rats with chronic left ventricular failure secondary to myocardial infarction5 or volume overload16 and from hamsters with hereditary cardiomyopathy.17 In addition, the marked contrast between ANF gene expression in nonfailing and failing human ventricular myocardia provides a molecular marker for human cardiac dysfunction.

The finding that steady-state levels of phospholamban mRNA are substantially decreased in failing human heart is consistent with studies of gene transcription in response to pressure overload and thyroid hormone in experimental animals.18 Our results differ from those of studies in experimental animals with hemodynamic overload in which there was induction of β-myosin heavy-chain mRNA.19 However, β-myosin heavy-chain gene expression has not been assessed previously in human ventricular myocardium. Furthermore, our results are consistent with investigations that fail to demonstrate myosin isozyme switches in hypertrophied human ventricle.20 Of importance is the fact that the data from this study support the hypothesis that cardiac dilatation resulting from idiopathic dilated cardiomyopathy is associated with selective changes in gene expression.

Summary

Our results demonstrate that quantitative PCR provides a tool with which to study the expression of genes known to be of functional importance in the human heart. The methodology is relatively rapid
and inexpensive and allows measurements to be performed in small quantities of tissue derived from intact heart. This technique can be applied to multiple genes of interest and may therefore be useful in increasing our understanding of the pathophysiology of heart muscle disease. Furthermore, molecular regulatory mechanisms in failing human heart appear to be gene-specific, and the selective increase in ANF gene expression in failing human heart provides a molecular marker of human heart disease.

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References

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