Viral Myocarditis
Identification of Five Differentially Expressed Genes in Coxsackievirus B3–Infected Mouse Heart

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Abstract—Differences in host susceptibility to viral myocarditis caused by a given strain of coxsackievirus B3 (CVB3) are known to be largely related to host genetic factors. Little is known, however, about the key genes that encode determinants (mediators) of myocarditis development or the nature of injury. To identify these genes and further understand the molecular mechanisms of the disease process, we have used a murine model and the differential display technique to fingerprint mRNAs from CVB3-infected mouse hearts. Total RNA was extracted from hearts of 4- and 10-week-old A/J(H-2a) mice at day 4 after CVB3 infection, and mRNAs were detected by reverse transcriptase–polymerase chain reaction and subsequently analyzed on polyacrylamide DNA sequencing gels. The differentially displayed bands were confirmed by Northern hybridization using the bands as cDNA probes. Twenty-eight upregulated or downregulated bands were selected from the sequencing gels; among these, 2 upregulated and 3 downregulated cDNA fragments were confirmed by Northern hybridization. DNA sequence analysis and GenBank searching have determined that 4 of the 5 candidate genes are homologous to genes encoding *Mus musculus* inducible GTPase, mouse mitochondrial hydrophobic peptide (a subunit of NADH dehydrogenase), mouse \(\beta\)-globin, and *Homo sapiens* cAMP-regulated response element binding protein (CREB) binding protein (CBP), respectively. The remaining candidate gene matches an unpublished cDNA clone, *M. musculus* Nip21 mRNA (GenBank accession number, AF035207), which is homologous to human Nip2, a Bcl-2 binding protein. Our data suggest preliminarily that both structural and nonstructural genes are involved in myocarditis development. For the structural gene, \(\beta\)-globin, we further confirmed its downregulation at the protein level by measuring the mean cell volume of red blood cells and found it was marginally reduced in the CVB3-infected group (\(P<0.06\)), with no change in hemoglobin concentration. Cardiac myoglobin concentration was also measured and found to be decreased (\(P<0.005\)), with a parallel decrease in total soluble protein in the CVB3-infected mouse myocardium (\(P<0.01\)). We also noted that the ratio of myoglobin to total protein was not significantly changed; this may be due to the downregulation of additional genes in the host heart, a number being observed on the differential display gels. The significant downregulation of \(\beta\)-globin major gene expression in the heart may be relevant to impaired cardiac function in both the early and late postinfection period. The other identified nonstructural genes are known to be involved in regulation of gene expression, signal transduction pathways, and apoptotic cell death. The altered expression of structural and nonstructural genes may play important roles in the mediation of myocarditis development and perhaps other pathological processes in the heart. (*Circ Res*. 1999;84:704-712.)

Key Words: myocarditis ■ differential mRNA display ■ coxsackievirus

Coxsackievirus B3 (CVB3) is a positive-sense, single-stranded RNA enterovirus. The CVB serogroup was ranked No. 1 in causation of clinical cardiovascular diseases by the World Health Organization in global surveillance of viral diseases from 1975 through 1985. Recent molecular characterization of isolates from community-acquired infections has reaffirmed the pathogenicity of CVB in human diseases. Furthermore, molecular diagnostic approaches have provided direct and compelling evidence of viral infection in the heart, pancreas, and other target organs. Viral genomic RNAs have been clearly shown in cardiac myocytes by in situ hybridization. Importantly, strong signals from hybridization of negative-strand viral RNA with sense riboprobes in heart tissue indicates that viruses are replicating within cardiac myocytes. In addition, our previous data and those of others have shown that virus infection can also cause extensive direct cell injury in the heart and other organs.
The susceptibility of certain host tissues and cells to CVB3 infection is influenced by many factors, such as sex, age, nutrition, pregnancy, genetic background, and epidemics; however, the most important factors are likely host genetic background and age. In the human population, not everyone is susceptible to CVB3 infection; despite a high attack rate, most people will never develop myocarditis in a lifetime. Similar observations have been made in animals. Different strains of mice have different levels of susceptibility to active CVB3 infection, and even those infection-susceptible animals with different genetic background will have distinctive target organ susceptibilities. For example, C57BL/6J mice are resistant to CVB3-induced myocarditis, but they develop severe hepatitis. A/J(H-2") and BALB/c mice can develop prominent myocarditis on viral infection but have a different likelihood of succumbing to liver injury. In addition to intrinsic myocyte factors responsible for disease, the immune system is also important in disease progression. It has been reported that T-cell populations in mice with different genetic backgrounds respond differentially to CVB3 infection, influencing the outcome of the disease.10,11,12
There are also data to invoke age as an important factor for host susceptibility to CVB3 infection. Infants are more susceptible to CVB3 infection than young children or adults. When human neonates acquire coxsackievirus infections at birth, the mortality is extremely high,13 and myocarditis may be severe. Neonatal and adolescent mice are susceptible to CVB3 infection and myocarditis, and the level of susceptibility decreases with increasing age.14-16 There are also many studies to show that gene-expression patterns underlying cardiac development change dramatically with maturation of the host.17,18 The age effect on myocarditis may be closely related to genetic background. In other words, a given age may be an important time point in the maturation of genetic expression in a host and as such may be a major determinant of disease.
Although the mechanisms of CVB3-induced myocarditis are not well understood, it is clear that the occurrence and progression of myocarditis depend on a complicated series of events involving interaction among genes of the virus and the host. Host susceptibility to CVB3 infection may in part reflect a parallel or sequential expression of certain genes encoding mediators of viral replication, viral persistence, and cell viability. These mediators may include proteins involved in signal transduction pathways, regulation of host gene transcription or translation, and structural integrity of the cell. During the CVB3 replicative cycle, virus-encoded proteases can disrupt important host factors, such as eukaryotic translation initiation factor 4G19 and TATA binding protein.20 This disruption of normal functioning of infected myocytes will ultimately affect the gene regulation of neighboring myocytes. The cumulative effect is dysfunction and death of infected myocytes and potential dysfunction and phenotypic alterations of other cardiac cells.
Much effort has been devoted to the identification of such regulatory genes; however, previous investigations have usually been confined to selected genes of interest and thus require information about gene sequences to design oligonucleotide primers or probes to measure such gene expression. Therefore, data obtained from studies of preselected genes reflect rather isolated phenomena in a pathogenetic picture that is particularly complex in vivo. To understand more definitively the mechanisms of this disease, we used the approach of differential mRNA display,21,22 systematically assessing gene expression at the transcription level in an A/J(H-2") mouse enteroviral model. This technique has allowed us to compare thousands of gene expressions from CVB3-infected and sham-infected mouse hearts side by side on DNA sequencing gels.
In this article, we report the identification of 5 candidate genes, the expressions of which were upregulated (2 genes) or downregulated (3 genes) in the early postinfection period in CVB3-infected A/J (H-2") mouse hearts. Of the 5 candidate genes, 1 is unknown, and 3 are nonstructural genes encoding enzymes or regulatory factors. The remaining modulated gene is β-globin. Downregulation of the β-globin major gene in heart tissue may reflect direct injury of myocardium. Identification of other nonstructural genes that are differentially expressed in infected heart may also provide new avenues to understanding molecular mechanisms of myocarditis and other pathological processes in CVB3-infected mice. The data presented here demonstrate that differential mRNA display (coupled with other defined end points) may accelerate our capacity to dissect the pathogenesis of viral myocarditis.

Materials and Methods
Animal and Tissue Processing
All experimental procedures conformed to the regulations of the Canada Council on Animal Care and were approved by the Institutional Animal Care Committee. Inbred adolescent (4-week-old) and young adult (10-week-old) A/J (H-2") mice (12 animals for each age, 6 mice per group) were infected with CVB3 (CG strain, 105 plaque-forming units) or sham-infected with PBS intraperitoneally and euthanized by CO2 narcosis on day 4 postinfection. The hearts were harvested, and biventricular transverse slices of tissue were used for plaque assays, in situ hybridization, and histopathology, respectively, to confirm the presence and locale of infection, the occurrence and nature of tissue injury, and the extent of inflammation in hearts with early myocarditis. A remaining portion was immediately frozen in liquid nitrogen and stored at −80°C for subsequent RNA extraction.

Viral Plaque Assay
HeLa cells were cultured in 6-well plates at 3.5×105 cells per well. When the cells were ∼85% to 90% confluent, serial dilutions of supernatant from the mouse cell lysate, prepared by homogenization, were inoculated into each well. One hour after infection, the inoculum was aspirated and cells in each well were overlaid with 2 mL of 0.7% warm agar containing 1:1 complete MEM with 10% FBS. After incubation at 37°C for 2 days, cells were fixed with Carnoy’s fixative and stained with 1% crystal violet solution for 5 minutes. The plates were washed, and the plaques were counted. Supernatant of cell lysates from sham-infected tissue was used as a control.

Histopathology
Transverse sections from the basal two thirds of the ventricular myocardium from each mouse heart, as well as transverse sections from each organ, were fixed in fresh 4% paraformaldehyde overnight, embedded in paraffin, sectioned, and stained with hematoxylin and eosin or with Masson’s trichrome. The severity of virus-induced disease was evaluated blindly by an experienced cardiovascular
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pathologist on a scale of 0 to 4 (least to most) for coagulation necrosis, contraction band necrosis, cytopathic effect, calcification, cellular infiltrates, stromal collapse, and fibrosis in a fashion similar to that previously described. This grading approach allows a clear distinction between mild, moderate, and severe disease.

In Situ Hybridization

In situ hybridization was performed on both agar-embedded cell preparations and tissue sections as previously described. Paraffin-embedded tissue sections were permeabilized with proteinase K, dehydrated, and hybridized using digoxigenin-labeled probe prepared by in vitro transcription according to the manufacturer's instructions. Hybridization was allowed to proceed at 42°C overnight followed by stringent washing in 50% formamide and 2X SSC. After anti-digoxigenin antibody was applied to the tissue sections, slides were enzymatically developed and incubated with color substrate overnight.

Isolation of Cellular RNAs and Northern Blot

Hearts from 4- and 10-week-old A/J (H-2a) mice, showing positive signals (infection) in the in situ hybridization and myocarditic lesions in the histopathologic staining, were selected for RNA isolation. Heart tissue (60 to 100 mg) was homogenized, and total cellular RNA was extracted with RNAzol B (Tel-Test) according to the manufacturer's instructions. Contaminating chromosomal DNA was eliminated by treating the sample with RNA-free DNase I at 37°C for 15 minutes, followed by phenol/chloroform extraction. Samples of total RNA (20 μg) were fractionated in 2.2 mol/L formaldehyde/1% agarose gels and transferred onto nitrocellulose filters. Specific probes were generated by labeling reamplified or cloned cDNA fragments with [α-32P]dCTP (random prime DNA labeling kit, Boehringer Mannheim). A GAPDH probe and ribosomal RNAs were used as loading control and mRNA size markers, respectively. After hybridization at 42°C overnight and a high-stringency wash at 60°C in 0.3 mol/L NaCl, 0.03 mol/L sodium citrate (pH 7.0), and 0.1% SDS, the filters were exposed to Kodak X-OMAT AR film for 3 to 7 days with intensifying screens.

Differential mRNA Display

Differential mRNA display was carried out as described, except that RNA samples were isolated from heart tissues rather than from cell cultures. CVB3-infected and sham-infected A/J (H-2a) mice at both 4 and 10 weeks of age were compared concurrently. Total RNA (0.5 μg) was reverse transcribed in a 50-μL reaction with modified 1-base anchored oligo-dT primers (GenHunter Corp). The first-strand cDNAs were then amplified by polymerase chain reaction (PCR) in the presence of an appropriate 3’ 1-base anchored oligo-dT primer, dNTPs, [α-32P]dATP, and an arbitrary 10-mer 5’ primer (RNAimage Kits, GenHunter). Forty PCR cycles were run with the following parameters: denaturation at 94°C for 30 seconds, annealing at 40°C for 2 minutes, and extension at 72°C for 30 seconds. In the control, water was substituted for cDNA. Labeled PCR products were analyzed by electrophoresis in 6% denaturing polyacrylamide gels. Reproducibility of amplification for selected bands was confirmed by repeating the reactions at least 3 times with different preparations of cDNA. Differentially upregulated bands were defined as those that were consistently present at higher density in virus-infected samples than in sham-infected samples. Differentially downregulated bands were defined as those that were consistently present at lower density in virus-infected samples than in sham-infected samples.

Identification of the Differentially Displayed mRNA

PCR bands of interest were excised from the gel and recovered by rehydration, boiling, and ethanol precipitation. The eluted cDNA was then reamplified using the same primers as those used in the differential display reaction but in the absence of isotope. The reamplified cDNA fragments were cloned directly into plasmid vector pCRII using the TA cloning kit (Invitrogen). Plasmid DNA of each clone was prepared following the standard method. Inserted cDNAs were isolated, radiolabeled, and used as probes in a Northern blot assay as described above. The cDNA fragments that generated a Northern hybridization signal were sequenced on an Applied Biosystems automated DNA sequencer using the Sanger et al26 dideoxy chain termination method. Nucleotide sequences obtained were compared with known sequences by searching the GenBank, EMBL, and EST (Expressed Sequence Tag) databases with the BLAST family of programs. In some cases, the translated amino acid sequences were also used to search the databases using the BLAST programs.

Measurement of Hemoglobin and Myoglobin

To further characterize the β-globin major gene and confirm its downregulation at the translational level, concentrations of hemoglobin and myoglobin in whole blood and in myocardium of CVB3-infected mice, respectively, were determined by a method described previously. Briefly, heart tissue was cut into small strips, frozen on dry ice, and homogenized using an electronic homogenizer. The proteins were extracted with phosphate buffer, 0.4 mol/L at pH 6.6. After centrifugation at 28 000g for 50 minutes, 3 mL of clear supernatant was mixed with 1 mL of 0.067 mol/L K2HPO4 and CO2 was then bubbled through the solution for 10 minutes. A pinch of Na2S·O3 was added, and the solution was rebubbled with CO2 for 5 minutes before optical density measurements were taken at wavelengths of 538 and 568 nm.

Statistical Analysis

All values are presented as mean±SEM. Statistical significance was evaluated using the Student t test for paired comparison, with P<0.05 considered statistically significant.

Results

Confirmation of Infection and Disease

To confirm CVB3 infection of heart muscle, in situ hybridization for positive- and negative-strand viral RNA in paraffin-embedded heart tissue sections was performed using digoxigenin-labeled sense and antisense RNA probes transcribed from CVB3 cDNA (Figure 1). There was significantly more viral RNA in the myocardium of 4-week-old mice than in that of 10-week-old mice. Negative-strand RNAs (Figure 1B and 1E) indicate the replication of CVB3 genomic RNAs in these cells. Pancreatic tissue sections (Figure 1C and 1F) from each mouse were hybridized with antisense strand probe to serve as a positive control. The pancreatic sections from 4- and 10-week-old mice, respectively, did not show as much age-related difference in amount of infection as seen in the heart, suggesting a cardiac-specific decrease in susceptibility of 10-week-old mice. To determine the viral titer in tissues, viral plaque assays were conducted using cell lysates. Infected heart tissues from 4-week-old and 10-week-old mice both produced plaques on the HeLa cell monolayer; however, tissue from 4-week-old mice produced significantly more plaques than that derived from 10-week-old mice, confirming previous reports. Histopathologic evaluation was also carried out to confirm the presence and severity of myocarditis in the hearts (Figure 2). Tissues from 4-week-old (Figure 2A) mice have more extensive myocyte injury (coagulation necrosis and cytopathic effect) than those from 10-week-old (Figure B) mice (statistical data not shown). These data confirmed previous observations. Panels C and D are age-matched sham-infected heart tissues used as a negative control.
Differential mRNA Display

To identify transcriptionally regulated genes of potential relevance to myocarditis development, we compared differential mRNA display patterns in hearts of 4- and 10-week-old mice infected with CVB3 with those of sham-infected animals at corresponding ages. PCR amplifications of reverse-transcribed first-strand cDNAs were conducted using a 3' anchored oligoT12N primer and a 5' arbitrary 10-mer primer.

Figure 1. In situ hybridization of CVB3-infected hearts (A, B, D, and E) and pancreas (C and F). Tissue sections of 4-week-old (A through C) and 10-week-old (D through F) A/J(H-2a) mice were hybridized with antisense strand (A, D, C, and F) and sense strand (B and E) RNA probes, respectively. Signals from negative strands of CVB3 in panels B and E suggest the replication of CVB3 in these cells. Two pancreas sections (C and F) were used as positive controls. Sham-infected mouse tissues of both ages were used as negative controls (data not shown). RNA probes were prepared by in vitro transcription in the presence of digoxigenin-dUTP. All tissues were harvested 4 days after CVB3 inoculation.

Figure 2. Histopathology of CVB3-induced myocarditis in A/J(H-2a) mice. CVB3-infected (A and B) and sham-infected (C and D) mouse hearts were harvested 4 days after inoculation from 4-week-old (A and C) and 10-week-old (B and D) mice. Tissues were processed and stained as described in Materials and Methods. Note the absence of pathological changes in hearts of sham-infected mice versus cardiac damage in hearts of CVB3-infected mice. Also note the decrease in myocyte injury and death in 10-week-old mice (B) as compared with 4-week-old mice (A).
Twenty-four primer combinations were applied to CVB3-infected and sham-infected samples. The obtained PCR products were analyzed on DNA sequencing gels. Figure 3 shows the mRNA display patterns with representative differentially expressed gene fragments, which are reproducible in repeat experiments. In total, we selected 28 bands that reflected upregulated or downregulated expression in CVB3-infected mouse hearts. Here, we selected 2 gels to show the altered expression for bands 2, 7, 12, and 15 (data not shown for other bands). Certain bands (e.g., band 2) demonstrated age-related differential expression in CVB3-infected mice. In other words, differential gene expression of certain genes is greater or lesser for given genes in 4-week-old mice than in 10-week-old mice.

Northern Hybridization

To confirm the differential gene expression patterns observed in gels, 28 altered bands were selected for further study. PCR fragments in the bands were recovered and reamplified by PCR (Figure 4) to make probes, which in turn were used to hybridize RNA blots prepared with 20 μg of total RNA from CVB3-infected or sham-infected hearts. Bands 8, 10, and 15 generated strong positive signals and also showed downregulation of transcription in CVB3-infected hearts as compared with sham-infected mice (Figure 5A). On the other hand, bands 2 and 7 generated strong positive signals and showed upregulation of transcription in CVB3-infected samples. Other bands did not produce Northern signals (data not shown) and were eliminated from further analysis in the present study. Such transcripts may not have been detected because their levels were below the sensitivity of the RNA blot analysis. The ribosomal RNAs and GAPDH were used as controls for RNA loading and gene transcriptional regulation. Relative positions of 18S and 28S ribosomal RNAs are indicated as size standards. The approximate transcript size for each gene is listed in the Table. The age-related differential expression of band 2 was also confirmed by Northern hybridization. Figure 5B shows that upregulation of gene expression for band 2 is significantly higher in 4-week-old mice than in 10-week-old mice. Ribosomal RNAs were used as a loading control.

Identification of Differentially Displayed Genes

Bands confirmed by Northern hybridization were reamplified by PCR and cloned into a TA cloning vector. The plasmid DNAs were sequenced by the Sanger et al.26 dideoxy chain-termination method. A GenBank search demonstrated that 4 PCR bands shared high sequence homology with respective known genes in the databases (Table), whereas band 10...
matches an unpublished cDNA clone, *Mus musculus* Nip21 mRNA (AF035207). Nip21 shares high sequence homology with human Nip2 (U15173). The GenBank accession numbers for these 5 clones (clones 2, 7, 8, 10, and 15) are AF071427, AF071428, AF071431, AF071429, and AF071430, respectively. The 4 known genes are described as follows: the cDNA fragment from clone 2, an upregulated gene, was found to be highly homologous to *M musculus* inducibly expressed GTPase (IGTPase). The 139-bp fragment is 98% identical to the published IGTPase sequence. Another upregulated cDNA fragment from band 7 shares 92% sequence homology with a mouse mitochondrial gene encoding a hydrophobic peptide, a subunit of NADH dehydrogenase. The remaining 2 candidates are downregulated genes. The first is from band 8 and has 95% sequence identity to mouse β-globin major gene. The second downregulated gene (gene 15) matches very well (88% identity) with human CREB-binding protein (CBP). CREB is a cAMP response element binding protein, which plays an important role in transcriptional initiation. CBP is a cotranscriptional factor of CREB.

**Cardiac Myoglobin Concentration**

To further assess the β-globin major gene and confirm the downregulation at the protein level, mean cell volume of red blood cells was measured and found to be marginally reduced in the CVB3-infected group (*P*, 0.06) with no change in hemoglobin concentration (data not shown). Cardiac myoglobin concentration was decreased (*P*, 0.005), with a parallel decrease in total soluble protein in the CVB3-infected mouse myocardium (*P*, 0.01) (Figure 6). We also found that the ratio of myoglobin to total protein was not significantly changed. Such concordance may be due to the downregulation of additional genes, which have been observed in the differential display gels.

**Discussion**

CVB3 infection causes remarkable alterations in host cellular physiology and morphology. This suggests that there is a complicated interaction between virus and host in disease development within the main target organs such as the heart. Until now, most postulates regarding mechanisms of cardiac myocyte injury and cardiac disease caused by CVB3 have been based on observations gleaned from histopathology, cell biology, virology, and immunology. Such studies have indicated that several host factors are pertinent to disease severity as induced by a given coxsackievirus. It is postulated that CVB3 infection of mice induces up- and downregulation of certain gene expressions encoding determinants (mediators) for heart disease development. These myocarditic determinants (known and unknown) may be produced by uninfected or by infected heart cells and/or infiltrating immune cells. Possible host determinants of disease include regulatory protein factors (activator or suppressor of gene expression) and host defense and immune mediators such as proteins related to interferon and major histocompatibility complex, metabolic proteins, and structural proteins, among many others, which serve as primary or secondary mediators of disease development. Modulation of gene expression lies at the center of regulatory mechanisms that control cellular responses, and thus intermediate and long-term dysregulation of certain genes is most likely critical to the etiology and progression of myocarditis. Therefore, comparisons of differential gene expression between virus-infected and sham-infected tissues have the potential of providing information of great pertinence to the pathogenesis of viral myocarditis.

Many methods have been developed to discern differential gene expression in one cell population or another. Subtractive hybridization is one such example; however, this technique

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**Figure 6.** Comparison of cardiac myoglobin concentrations in myocardium of CVB3-infected and sham-infected mice. Myoglobin was prepared and measured according to the method of Reynafarje. The difference in optical density at 538 and 568 μm was multiplied by the factor 117.3, and the resulting value expressed the concentration of myoglobin in milligrams per gram of wet tissue. Data are mean ± SEM. *P* < 0.005; **P** < 0.01.
gives incomplete recovery and selects only for either underexpressed or overexpressed genes. Furthermore, the screening is laborious. Differential mRNA display, which we used in this study, can overcome these limitations. This method has been used to study many diseases. In studies of cancer, pathogenesis comparisons were made between 2 populations of in vitro cell lines at once. Our approach involved the comparison of gene expression patterns derived from RNAs isolated from a whole organ, the heart. This latter approach preserved the entire pathophysiologic environment associated with the disease process of myocarditis.

Using 24 primer combinations, we identified 28 differentially expressed cDNA bands that were reproducibly upregulated or downregulated in CVB3-infected mouse hearts for both 4- and 10-week-old animals. Northern hybridization, DNA sequencing, and GenBank searching further identified 5 of the 28 bands as genes encoding IGTPase, mitochondrial hydrophobic peptide (a subunit of NADH dehydrogenase), β-globin, CBP, and an unpublished cDNA clone homologous to human Nip2, respectively.

One of the goals of this study was to develop strategies for identification of host determinants responsible for myocarditis initiation and progression toward altered myocyte phenotype. As mentioned earlier, these mediators may be primary or secondary factors in the disease process. The link of the 5 identified genes to CVB3-induced myocarditis is especially interesting, because both structural and nonstructural genes were found to be involved. Typically, the structural genes are not considered as important as nonstructural genes in disease development. This may be not true for all settings. Recently, 2 structural genes encoding dystrophin and muscle LIM protein have been implicated in dilated cardiomyopathy, a common heart disease that has been suggested to result from long-term infection of CVB3. Modulation of the gene encoding β-globin in viral myocarditis provides a new suggestion that metabolically active structural genes have importance in heart disease occurrence. To further determine the relationship between the downregulation of β-globin and the myocardium injury, we measured the concentration of hemoglobin in blood and of myoglobin in the heart tissue from CVB3-infected mice. Data showed that the concentration of hemoglobin did not change with myocarditis. However, the concentration of cardiac myoglobin did show a significant decrease, with a parallel decrease in total soluble proteins. This suggests that the downregulation of β-globin major gene may relate to the decrease of cardiac myoglobin concentration. We also noted that the ratio of myoglobin to total protein was not changed in the CVB3-infected group; this may be due to the downregulation of other genes (primary or secondary responses), which have been observed on gels of differential mRNA display. Although many genes showed decreased expression, the downregulation of β-globin major gene was most visible and reproducible as compared with other bands. As is known, myoglobin is a cytosolic heme-protein selectively expressed in cardiac and skeletal myocytes, in which it functions to augment delivery of oxygen for mitochondrial respiration during heavy contractile work. Therefore, downregulation of this gene expression may cause severe damage of the myocardium. These findings provide support at the molecular level for the importance of direct CVB3-induced injury of cardiac myocytes.

IGTPase is representative of a newly identified group of interferon-γ-induced GTPases, the functions of which are poorly understood. Recent reports indicate that IGTPase is located predominantly in the endoplasmic reticulum of cells; however, GTP binding status of IGTPase is independent of its capacity to localize in the cellular compartment. Thus, the function of IGTPase may involve protein processing or trafficking. It has been known that interferon-γ is a pleiotropic cytokine that regulates a variety of immunological and inflammatory responses in fighting diseases. Interferon-γ, however, is also thought to be involved in many pathological responses (eg, in multiple sclerosis), and its production exacerbates disease symptoms and increases relapse rate. In CVB3-infected hearts, overexpression of interferon-γ by invading immune cells would in turn induce the production of IGTPase in the heart. The upregulated IGTPase gene expression may trigger signals attempting to stimulate protein synthesis, process, and trafficking. The consequences of such signaling may include maintenance of myocyte function in the face of a destructive insult and, in theory, could contribute to postinfectious hypertrophy.

A third gene is homologous to a mitochondrial gene encoding a hydrophobic peptide (ND1), a subunit of NADH dehydrogenase. It has been known that NADH dehydrogenase is an important enzyme in the tricarboxylic acid cycle. The link between this gene and viral myocarditis is particularly interesting, because mitochondria serve as the center for energy production via the tricarboxylic acid cycle and thus as the major power source for contractility of the heart. Altered expression of NADH dehydrogenase in mitochondria of muscle cells may directly affect the function of the heart. In addition, it is increasingly reported that mitochondrion is a critical organelle involved in the initiation of apoptosis. Cytochrome c release from mitochondrial membrane can activate caspases and in turn induce apoptotic cell death. NADH dehydrogenase subunit is a hydrophobic peptide embedded within the mitochondrial membrane. Upregulation of this gene may alter the membrane permeability and affect the release of mitochondrially encoded factors. Whether the altered expression of NADH dehydrogenase is related to the induction of apoptosis remains to be determined.

A fourth gene encodes a nuclear protein, CREB binding protein (CBP), that can bind to phosphorylated CREB to serve as a cofactor in the regulation of transcription of target genes. CREB is a basic leucine-zipper nuclear transcription factor and may be involved in the development of dilated cardiomyopathy. The activity of CREB is primarily regulated by protein kinase A–mediated phosphorylation of Ser133. Activated CREB can bind to the sequence motif known as the cAMP response element and participate in cAMP-regulated gene expression. Phosphorylation of CREB at Ser133 specifically enhances its binding ability to CBP. There is also evidence that CBP cooperates with upstream activators, such as c-jun, that are involved in mitogen response transcription. Therefore, as a cofactor, CBP is recruited to the promoter through interaction with certain phosphorylated factors. CBP may play a critical role
in the transmission of cAMP-induced signals from cell surface receptors to the transcriptional apparatus.

We predicted that the mediators of host susceptibility to CVB3 infection would include factors involved in signal transduction. CBP is indeed such a factor in the cAMP/protein kinase A pathway. Although the precise mechanism by which viral infection induces myocarditis via downregulation of CBP is not clear at present, the observation of downregulation of a vital cofactor, CBP, in cAMP-induced transcription has provided clues as to why the viral infection can shut down or inhibit host protein synthesis. It is clear to some degree that if gene expression is regulated through a cAMP/protein kinase A pathway (eg, the phosphorylation of transcription factor CREB must be catalyzed by protein kinase A), its transcription will be inhibited by CVB3 infection. If such altered gene expression, in turn, regulates cardiomyocyte metabolism or produces structural proteins in the heart, such as cardiac myoglobin, viral infection will cause myocardial injury both physiologically and structurally. We found that there were more downregulated than upregulated bands on the differential display gels. This can be attributed in part to the inhibition of key factors and cofactors (eg, CBP) involved in cAMP-regulated gene transcription and to the known inactivation of translation initiation factor eIF4G by enteroviruses.52

The remaining candidate gene, mouse Nip21, may be the most interesting gene among these 5 candidates in the context of CVB3-induced cell death. First, this gene product is homologous (66% similarity in 126 amino acid region) to the GTPase-activating protein, RhoGAP,50 which raises the possibility that Nip21 is involved in signal transduction pathways. Second, Nip21 shows high sequence homology to the human Nip2 protein. This human protein is capable of interacting with the apoptosis regulator Bcl-2 and a homologous protein, the adenovirus E1B 19-kDa protein.53 Mutational analysis indicated that this human Nip2 does not interact with 19-kDa mutants defective in suppression of cell death.54 Thus, the human isologue (Nip2) of Nip21 may be a mediator interacting with Bcl-2 or E1B 19-kDa protein to promote cell survival. Downregulation of Nip21 by CVB3 infection in the heart may therefore promote myocyte cell death, an early feature of CVB3-induced disease.7 Furthermore, CVB3 has the potential to activate caspases in infected cells.54 Such regulation of cell death proteins may be an important early feature after myocardial infection before immune infiltration, as well as later.

With the identification of at least 5 candidate determinants of CVB3-induced myocarditis, we have obtained new clues to the molecular pathogenesis of CVB3 myocarditis. Meanwhile, we also affirmed the value of differential mRNA display analysis as a tool to provide insights into molecular mediators associated with complex processes. In the case of CVB3 myocarditis, we have used an established animal model instead of cell lines, providing a real disease-environment assessment. The present study only focused on gene responses at day 4 after infection. Later time points of study at days 14 to 21 after infection (and later) may identify more gene expression related to the immune response and reparative processes, as well as other secondary gene responses. It has been suggested that a subset of dilated cardiomyopathy is the late phase of CVB3 myocarditis.55 Thus, studies at even later time points may reveal genes responsible for myocardium hypertrophy and myocardial remodeling. With progress of our future studies at different time points, more candidate genes, upregulated and downregulated, known and unknown, are being identified and characterized, particularly as they relate to cardiac myocyte injury and impairment. An integrated picture of molecular pathogenesis of this important form of heart disease will be required before effective therapies can be developed.

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