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Differential Expression of c-jun and junD in End-Stage Human Cardiomyopathy

Pia S. Pollack,* Lisa M. Pasquarello, Ricardo Budjak, Eduardo Fernandez, Kenneth J. Soprano, Brian G. Redfern, and Bruce Goldman

Departments of Medicine, Microbiology and Immunology, and Pathology, Temple University School of Medicine, Philadelphia, Pennsylvania

Abstract The proto-oncogenes c-jun and junD are closely related transcriptional factors with opposing actions on cell growth and division. Expression of c-jun rapidly increases as cells enter the cell cycle. Levels of c-jun are also increased in the early stages of experimental cardiac hypertrophy and failure but expression decreases with time. In contrast, junD accumulates in quiescent cells. Expression in end-stage cardiomyopathy has not been studied. Steady-state levels of c-jun and junD mRNA were determined in failing human myocardium (obtained at the time of cardiac transplantation) and in control myocardium from patients who died of noncardiac causes. Relative expression was normalized for glyceraldehyde-3-phosphate dehydrogenase expression. Levels of junD were almost four-fold depressed in myocardium from myopathic hearts (2.1 ± 0.27, $x \pm SE$; $n = 20$) vs. the controls (7.7 ± 1.1; $n = 3$). Levels of c-jun were similar in both myopathic and control hearts. Relative expression of beta-myosin heavy chain was the same in both myopathic and control hearts. Levels of junD were still found to be depressed in the myopathic hearts after normalization for myosin heavy chain gene expression. We conclude that c-jun and junD are differentially regulated in end-stage human cardiomyopathy with expression of junD being decreased while relative levels of c-jun mRNA remain unchanged. Further studies are needed to determine the role of junD down-regulation in the development and/or maintenance of the abnormalities present in end-stage heart disease. J. Cell. Biochem. 65:245–253.

Key words: c-jun; junD; cardiomyopathy; myosin; gene expression

The jun family of transcriptional regulatory proteins share a high degree of sequence homology but have opposing actions on cell growth and division. Expression of c-jun rapidly increases when cells are stimulated to enter the cell cycle (Ryder and Nathans, 1988). In contrast to c-jun, junD mRNA levels are generally higher in fully differentiated and quiescent cells with junD transcription being relatively unaffected by mitogenic stimuli (Ryder et al., 1989). Members of the jun family combine to form either a heterodimer with a member of the Fos family or dimerize among themselves to form a jun-jun homodimer. These Fos-jun and jun-jun complexes are referred to as the AP-1 transcription factor and regulate transcription by binding to specific DNA consensus sequences (Curran and Franza, 1988; Ryseck and Bravo, 1991). The levels of specific Fos-jun and jun-jun complexes vary as a function of cell type and the specific cellular environment (Curran and Franza, 1988). junD-junD dimers predominate in nonproliferating G_0 cells (Pfarr et al., 1994) but junD is not a component of the AP-1 complexes present in proliferating cells (Kovary and Bravo, 1991). These observations suggest that c-jun and junD are active under different physiological conditions and that expression of junD plays a role in the maintenance of the quiescent state.

It is generally believed that myocytes hypertrophy in response to stress but do not increase in number (Chien et al., 1991). Nevertheless, levels of c-jun mRNA rapidly increase in response to elevations in myocardial wall stress but expression of c-jun then decreases over time (Schunkert et al., 1991). Levels of c-jun also increase in stretched myocytes in vitro in association with the induction of a nuclear factor that binds specifically to the AP-1 consensus sequence (Sadoshima et al., 1992). Increased

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*Correspondence to Dr. Pia S. Pollack, Section of Cardiology, Temple University School of Medicine, 3400 North Broad Street, Philadelphia, PA 19140.

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levels of c-jun mRNA also are also seen when myocyte hypertrophy develops in response to stimuli such as catecholamines [Iwaki et al., 1990] or to angiotensin II [Sadoshima and Izumo, 1993]. Overexpression of c-jun directly transactivates the skeletal α-actin promoter [Bishopric et al., 1992] suggesting a direct role for the Fos-jun complex (AP-1) in the regulation of at least some components of the hypertrophic response.

The purpose of this study was to characterize the expression patterns of c-jun and junD in normal and diseased adult human ventricular myocardium. The patterns of c-jun expression have been well characterized in the myocardium in vivo and in vitro [reviewed in Komuro and Yazaki, 1993, and in Pollack, 1995]. Little information is available in regards to patterns of junD expression in the intact heart; a very transient increase in junD mRNA levels in short-term cultures of hypoxic neonatal myocytes has been described [Webster et al., 1993]. Because junD is expressed at higher levels in differentiated and quiescent cells [Ryder et al., 1989], we hypothesized that, in contradistinction to c-jun and c-fos, junD would be expressed in normal adult myocardium. End-stage cardiomyopathy is characterized by myocyte hypertrophy and interstitial proliferation [Beltrami et al., 1995; Boluyt et al., 1994; Weber et al., 1994] and it appears logical that myocardial levels of junD would be down-regulated in that setting.

METHODS

Patients

Human myocardium was obtained from patients in end-stage heart failure at the time of cardiac transplantation. The tissue was immediately snap-frozen in liquid nitrogen and stored at −70°C until the time of analysis. Snap-frozen myocardium from patients without a history of heart disease, hypertension, diabetes, or exposure to chemotherapeutic agents was obtained from The National Disease Research Interchange (NDRI).

Northern Blot Analysis

RNA was isolated from ventricular tissue by the guanidinium isothiocyanate method [Chomczynski and Sacchi, 1987]. Total RNA (20 μg) was separated on 2.2 M formaldehyde/1.2% agarose gels and transferred to nitrocellulose. Northern analysis was performed according to standard methods [Sanbrook et al., 1989]. Prehybridizations and hybridizations were done at 42°C in 50% formamide. Probes were labeled with ^{32}P deoxycytidine 5′-triphosphate by the random primer method [Feinberg and Vogelstein, 1983].

cDNA probes were a 400 base pair Pst I fragment of human c-jun [Angel et al., 1988] and a 1.7 kb Eco RI insert of murine junD [Ryder et al., 1989]. For analysis of beta-myosin heavy chain gene expression, a 162 bp cDNA fragment, which encodes parts of exons 9 and 10 of the human beta-myosin heavy chain gene, was obtained from a human cardiac muscle cDNA library and cloned into Bluescript. To normalize for variability in loading, all blots were also probed with a 780 bp Pst I/Xba I fragment of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [Tso et al., 1985] obtained from the American Type Culture Collection, Rockville, MD.

Filters were washed three times for 5 min each in 2 × SSC (150 mM NaCl, 15 mM Na citrate, 0.1% SDS) at 23°C, then three times for 30 min each in 0.1 × SSC at 52°C. Autoradiographs were exposed at −70°C with intensifying screens. Exposure time was 96 h for c-jun and junD, 72 h for beta-myosin heavy chain, and 4 h for GAPDH. Blots were scanned by laser densitometry. The signal from each sample was normalized to the signal obtained by probing with GAPDH.

Statistics

Values were reported as means ± SE. Statistical differences between two groups were evaluated by the unpaired Students' *t*-test. Comparisons between three groups were evaluated with a one-way analysis of variance followed by the Student-Newman-Keul's multiple comparisons test (InStat GraphPad Software Inc., San Diego, CA). A P value of <0.05 was considered significant.

RESULTS

Patient characteristics. Table I summarizes pertinent clinical information on the patients whose myocardium was studied. Myocardium was obtained from 20 patients, 18 of whom were male, averaging 49 ± 2.3 years in age. Pathological diagnoses were available for 15 of 20 patients; diagnoses were established on clinical grounds (including cardiac catheterization) in the remainder. Approximately half the patients had ischemic heart disease; most of the rest were diagnosed with idiopathic cardiomy-

opathy. Control hearts were obtained from patients without a history of heart disease, hypertension, diabetes, or cancer treated with cardiotoxic agents. The patients with cardiomyopathy were similar in age to the control patients (57.0 ± 4.0 years, P = NS). All control hearts were from female patients.

Levels of junD mRNA are decreased in myopathic hearts while expression of c-jun is unchanged. Figure 1 shows a representative blot that was sequentially probed with junD, c-jun, and GAPDH. The junD signal was increased in the RNA from the control myocardium relative to the myopathic hearts. In contrast, c-jun was present at very low levels in both end-stage myopathic and control hearts. These hybridization signals were quantitated with laser densitometry. Figure 2 shows relative levels of junD mRNA and c-jun mRNA in myopathic and control hearts normalized for GAPDH expression. Expression of junD was depressed almost four fold in the myopathic as compared with the control hearts. This difference is highly significant ($P < 0.00001$). There were no significant differences in c-jun expression between the myopathic and control hearts.

junD mRNA is down-regulated in both ischemic and nonischemic cardiomyopathy. End-stage cardiomyopathy may be secondary to ischemic heart disease or due to other causes. Expression of c-jun and junD was compared to control levels in those patients whose cardiomyopathy was secondary to an ischemic etiology and patients in whom there was no evidence of ischemic heart disease. Figure 3 demonstrates that down-regulation of junD expression in end-stage heart disease is a consistent finding regardless of etiology. Steady-state levels of junD mRNA were slightly lower in the nonischemic vs. the ischemic hearts but this difference did not reach statistical significance.

TABLE I. Patient Characteristics

Age:	49 ± 2.3 years
	(controls, 57 ± 4.0 years; P = NS)
Sex:	18M/2F
	(controls, 3F)
Diagnosis:	9 ischemic cardiomyopathy
	1 ischemic/valvular cardiomyopathy
	7 idiopathic cardiomyopathy
	1 active myocarditis
	1 rheumatic carditis
	1 transplant myopathy

The pattern of junD expression in myopathic hearts is unlikely to be due to changes in myocyte content. Fibrosis is present in failing hearts and patients with ischemic myopathies may have large areas of scar [Weber et al., 1992; Boluyt et al., 1994; Beltrami et al., 1995]. Care was taken to isolate RNA from areas of myocardium that appeared grossly normal but it is possible that the altered expression of junD in myopathic hearts might be attributable to an alteration in the cellular composition of the myocardium. Expression of beta-myosin heavy chain mRNA was therefore examined as an index of myocyte content in seven of the myopathic hearts and in the three normal controls. Figure 4 shows a blot that was probed for beta-myosin heavy chain and then with GAPDH to assess RNA loading. Average steady-state levels of myosin heavy chain mRNA expression (normalized for GAPDH expression and expressed in arbitrary units) did not differ between the myopathic (1.40 ± 0.21) and control hearts (2.91 ± 2.27; P = NS) although there was some scatter in the control group. Nevertheless, normalizing for myosin heavy chain content did not affect relative junD expression. Steady-state levels of junD mRNA were still significantly depressed in the myopathic hearts relative to the controls after correcting for myosin heavy chain mRNA expression (Fig. 5).

DISCUSSION

This is the first demonstration that junD mRNA is present in human myocardium and that its expression is altered in pathophysiologic states. Members of the jun family as well as other proto-oncogenes are rapidly, but only transiently, up-regulated in experimental models of hemodynamic load [Izumo et al., 1988, Pollack et al., 1994], catecholamine stimulation [Kolbeck-Ruhmkorff and Zimmer, 1995], or hypoxia [Webster et al., 1993; reviewed in Das et al., 1995]. Other studies have established that AP-1 complexes directly up-regulate gene expression in the early stages of the hypertrophic response. Overexpression of c-jun directly transactivates the skeletal α-actin promoter [Bishopric et al., 1992] and overexpression of c-jun results in the dose-dependent induction of the human atrial natriuretic peptide (ANP) gene promoter via binding to a TRE-like *cis*-acting regulatory sequence [Kovacic-Milivojevic and Gardner].

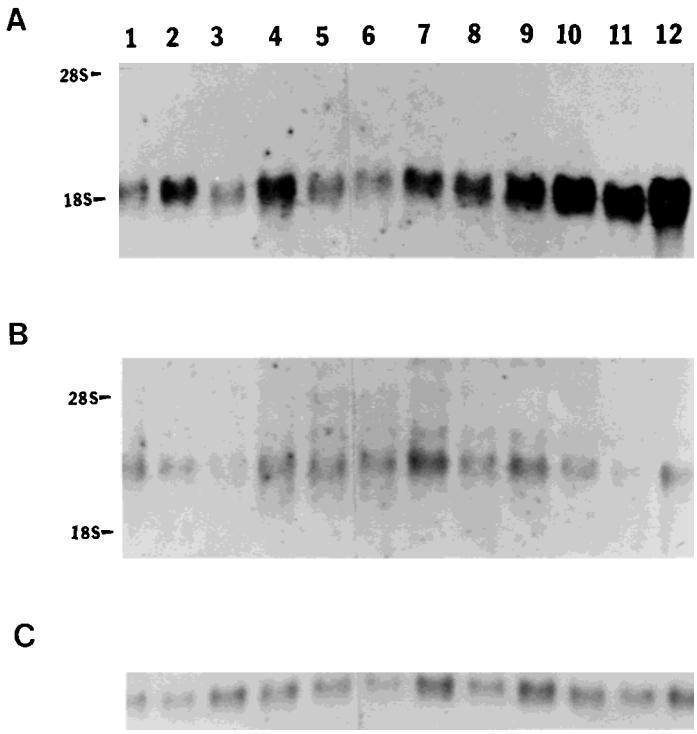

Fig. 1. Northern blot of myopathic **(lanes 1–9)** and control **(lanes 10–12)** human myocardium. The blot was sequentially probed with junD **(A),** c-jun **(B),** and GAPDH **(C).**

It has been inferred that similar phenomena are operative in clinical disease. This has been difficult to prove as human myocardial tissue is generally not available for study until the late stages of a disease process. The proto-oncogene junD was chosen for study because, unlike other members of the jun family, it is expressed at higher levels in differentiated and quiescent cells [Ryder et al., 1989]. We hypothesized that, in contrast to c-jun and members of the fos family, junD should be present in normal myocardium. In accordance with our hypothesis, junD was detected in normal human myocardium and steady-state mRNA levels were depressed almost four-fold in end-stage cardiomyopathy. Decreased junD expression remained a consistent finding independent of the etiology underlying the development of heart failure in these patients. Not surprisingly, we were unable to detect c-fos in either control or myopathic myocardium (data not shown) and c-jun was detectable only at very low levels in both control and myopathic hearts.

The results of this study suggest that alterations in the relative expression of different jun proteins may be a mechanism by which gene expression can be regulated in the failing heart. Members of the Fos and jun protein families dimerize to form the AP-1 transcription factor [Curran and Franza, 1988] which interacts with DNA binding sites. Fos-jun heterodimers and jun-jun homodimers can be composed of any member of the jun family as all are capable of forming functionally active AP-1 transcription complexes [Curran and Franza, 1988; Ryder et al., 1989]. Different Fos-jun and jun-jun transcription complexes should not, however, be considered functionally interchangeable with respect to DNA binding interactions. c-jun, junB, and junD proteins bind with entirely different affinities to synthetic oligonucleotides that have identical AP-1 or CRE consensus sequences but diverge in their flanking regions [Ryseck and Bravo, 1991]. Thus, the DNA sequences adjacent to the DNA binding site will influence the binding and stability of a particular AP-1 protein-DNA complex. The relative proportions of c-jun, junB, junD, and Fos-related proteins within the cell vary in a defined fashion in response to different environmental cues and this provides a mechanism for the precise modulation of AP-1 regulated gene transcription [Kovary and Bravo, 1991].

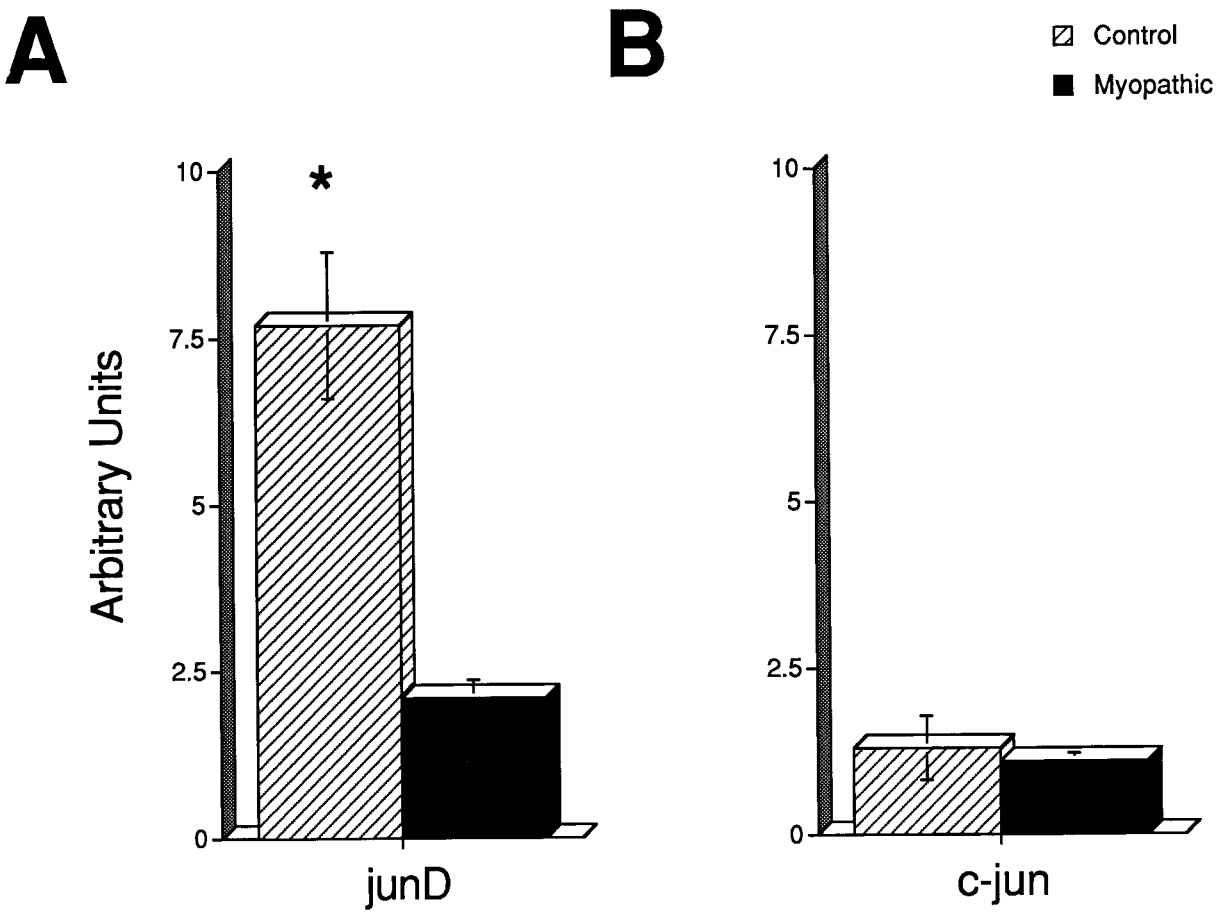

Fig. 2. Relative levels of junD mRNA **(A)** and c-jun **(B)** in control (n = 3) and myopathic (n = 20) hearts. Results are expressed in arbitrary units and are normalized for GAPDH expression. *$P < 0.00001$.

A decrease in junD levels within the myopathic heart would alter the relative levels of the various AP-1 components thereby affecting AP-1 regulated gene transcription. Alterations in the composition of AP-1 components have been shown to occur in other cellular adaptive processes. During replicative senescence of normal human fibroblasts, c-fos and junB mRNA levels decline while c-jun expression remains constant [Irving et al., 1992]. This results in a predominance of jun-jun homodimers relative to the Fos-jun heterodimers that are present during earlier passages of fibroblast cell lines [Riabowol et al., 1992] with changes in the relative levels of various AP-1 dimer components being associated with the growth inhibition seen in late passage cells.

Our data suggests that junD down-regulation in the myopathic heart may alter AP-1 complex composition resulting in changes in binding to specific target sequences. In turn, this may modulate, either directly or indirectly, the expression of specific cardiac genes. Possibly, this occurs via ras mediated pathways. Overexpression of junD negatively regulates fibroblast growth and partially suppresses transformation by an activated ras gene [Pfarr et al., 1994]. Activation of ras-dependent intracellular signaling pathways has been directly implicated in the pathogenesis of cardiac hypertrophy in vitro [Thornburn et al., 1993] and in vivo [Hunter et al., 1995]. In the latter study, mice in which a ras transgene was expressed within the ventricle displayed morphological and physiological markers of cardiac hypertrophy. Down-regulation of junD in myopathic myocardium may then be associated with the activation of ras mediated pathways that lead to the development of the hypertrophic phenotype.

As expected, c-jun mRNA was barely detectable in both normal and end-stage myopathic hearts. While c-jun mRNA is up-regulated in experimental models of cardiac hypertrophy in vivo and in vitro [reviewed in Komuro and

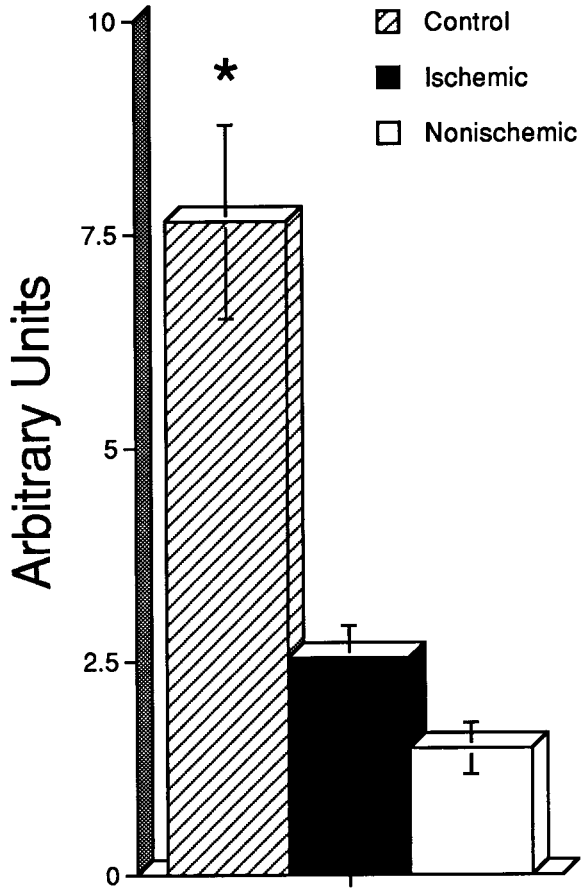

Fig. 3. Relative levels of junD mRNA in myocardium from controls, patients with ischemic cardiomyopathy, and nonischemic cardiomyopathy. Results are expressed in arbitrary units and are normalized for GAPDH expression. *$P < 0.001$ control myocardium vs. ischemic or nonischemic myocardium.

Yazaki, 1993, and in Pollack, 1995], these findings are only present soon after the onset of the hypertrophic stimulus with levels rapidly returning to baseline. While it is possible that c-jun might be up-regulated in the interstitium during the development of cardiac failure in vivo, chronic up-regulation of c-jun has not been found in either experimental models of heart failure nor in the human hearts studied here.

Other mechanisms, in addition to the relative proportions of jun and Fos family members, affect AP-1 transcriptional activation. Phosphorylation of serine residues within the c-jun transactivation domain by mitogen-activated protein (MAP) kinase or c-jun NH_2 terminal kinases (JNKs) enhances c-jun transcriptional activity [Pulverer et al., 1991; Hibi et al., 1993; Kyriakis et al., 1994] and these mechanisms participate in the myocardial response to stress. Mechanical activity activates MAP kinase in isolated myocytes [Yamazaki et al., 1993]; both MAP kinase and JNK are activated in response to ischemia and reperfusion injury [Knight and Buxton, 1996].

A recently identified novel group of coactivators increases the specificity of target gene activation by AP-1 proteins [Claret et al., 1996]. JAB1 is a nuclear protein that stabilizes complexes of c-jun or junD with AP-1 binding sites but does not alter the binding of either junB or v-jun to these very same sites. Possibly, JAB1 or a related protein contributes to the specificity of AP-1/regulatory sequence interactions in response to physiologic and patholophysiologic cues within the heart.

Although we were able to study a relatively large number of human hearts with end stage disease ($n = 20$), only three control hearts were available for study. Nevertheless, the differences in junD expression were highly significant between the two groups. These differences remained significant even when corrected for myosin heavy chain expression so it is unlikely that the decrease in junD expression can be attributed to any factor other than the presence of end-stage cardiac myopathy in the experimental group. The differences in junD expression cannot be due to differences in age as the control hearts were obtained from patients of similar age as the patients undergoing cardiac transplantation. The control hearts were all obtained from female patients. Although only two of the transplanted patients were female, it seems unlikely that gender would play a role in junD expression as normalized junD levels for the two female patients were actually lower (1.96 and 1.28) than for the group of 20 patients as a whole (2.1 ± 0.27). While control tissue was not obtained from patients known to have either cardiac disease or other abnormalities (such as hypertension or diabetes) that are associated with the development of myocyte hypertrophy, it is always difficult to determine if the controls were truly "normal." However, the presence of occult disease in the control hearts would have been expected to diminish differences between the two groups rather than accentuate them.

The cell type(s) expressing junD were not directly assessed in this study but, as discussed above, the results were not affected by normalizing junD expression to that of a muscle specific gene (myosin heavy chain). Although there was variability in myosin heavy chain gene expression from sample to sample in control

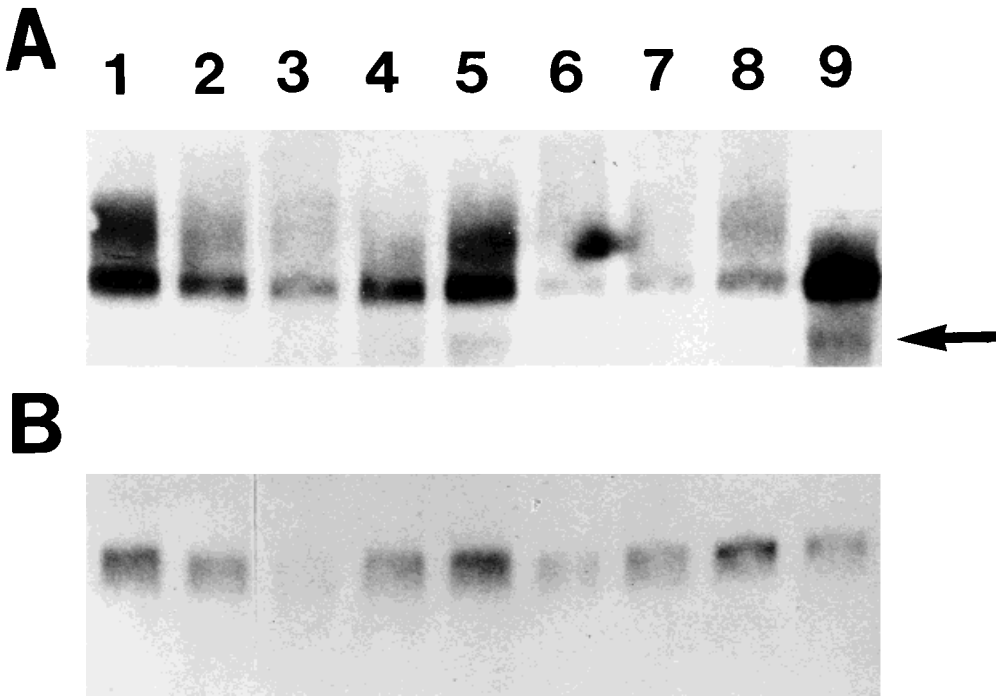

Fig. 4. Northern blot of myopathic **(lanes 1–6)** and control **(lanes 7–9)** human myocardium. The blot was sequentially probed with a beta-myosin heavy chain **(A)** and GAPDH **(B).** The position of the 28S subunit on the beta-myosin heavy chain blot is indicated by the arrow.

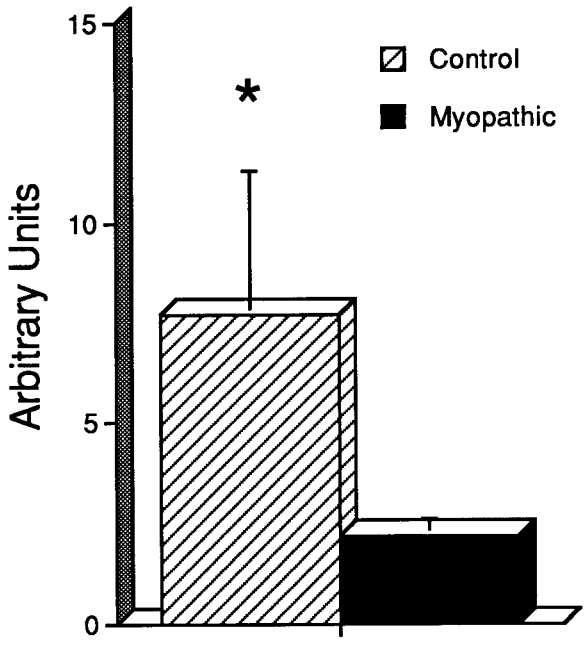

Fig. 5. Levels of junD mRNA in control (n = 3) and myopathic (n = 7) hearts relative to myosin heavy chain expression. Results are expressed in arbitrary units and are normalized for GAPDH expression. *$P < 0.05$.

and myopathic hearts, average myosin heavy chain expression was not different between the two groups. The observed differences in junD expression cannot be due to alterations in the cellular composition of the myocardium or to sampling from areas with significant fibrosis.

The results of this study demonstrate that junD and c-jun are differentially expressed in normal and failing human myocardium suggesting that the balance of AP-1 transcriptional factor components is altered in the human myopathic heart. Changes in AP-1 composition would affect the transcription of structural cardiac genes resulting in alterations in cardiac function. In theory, the first part of this hypothesis could be directly tested by studying the composition and binding of AP-1 transcriptional complexes in failing and control myocardium. Unfortunately, these experiments were not possible due to technical considerations related to the availability and preservation of clinical material. Further studies, using animal models of hypertrophy and failure and cultured cardiac myocytes, will be needed to define the role of junD down-regulation in the development and/or maintenance of the abnormalities present in end-stage heart disease.

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