Coordinated Post-transcriptional Regulation of Hsp70.3 Gene Expression by MicroRNA and Alternative Polyadenylation

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Heat shock protein 70 (Hsp70) is well documented to possess general cytoprotective properties in protecting the cell against stressful and noxious stimuli. We have recently shown that expression of the stress-inducible Hsp70.3 gene in the myocardium in response to ischemic preconditioning is NF-κB-dependent and necessary for the resulting late phase cardioprotection against a subsequent ischemia/reperfusion injury. Here we show that the Hsp70.3 gene product is subject to post-transcriptional regulation through parallel regulatory processes involving microRNAs and alternative polyadenylation of the mRNA transcript. First, we show that cardiac ischemic preconditioning of the in vivo mouse heart results in decreased levels of two Hsp70.3-targeting microRNAs: miR-378* and miR-711. Furthermore, an ischemic or heat shock stimulus induces alternative polyadenylation of the expressed Hsp70.3 transcript that results in the accumulation of transcripts with a shortened 3'-UTR. This shortening of the 3'-UTR results in the loss of the binding site for the suppressive miR-378* and thus renders the alternatively polyadenylated transcript susceptible to miR-378*-mediated suppression. Results also suggest that the alternative polyadenylation-mediated shortening of the Hsp70.3 3'-UTR relieves translational suppression observed in the long 3'-UTR variant, allowing for a more robust increase in protein expression. These results demonstrate alternative polyadenylation of Hsp70.3 in parallel with ischemic or heat shock-induced up-regulation of mRNA levels and implicate the importance of this process in post-transcriptional control of Hsp70.3 expression.

Heat shock (HS)$³$ and the subsequent expression of heat shock proteins have long been known to possess cytoprotective properties and provide cardioprotection against ischemia/reperfusion (I/R) injury (1–3). The Hsp70 family is among the best studied of the heat shock proteins, has been shown to be induced by many cardiac preconditioning stimuli, and plays a necessary role in the second window of protection (3–6). Hsp70.1 and Hsp70.3 are two nearly identical stress-inducible Hsp70 genes present in the murine heart. The two protein products differ by only a single amino acid, but curiously, the sequence of the two genes is divergent in the regulatory regions of the gene promoter (beyond the first 270 amino acids proximal to the transcriptional start site) and within the 3'-untranslated region (3'-UTR) of the mRNA transcript. Historically, these two genes are considered to be functionally redundant.

We have recently shown that NF-κB-dependent expression of heat shock protein 70.3 (Hsp70.3), but not the closely related Hsp70.1, is necessary for the late phase or second window of ischemic preconditioning (IPC) cardioprotection against acute I/R in the heart (6). In fact, Hsp70.3 and Hsp70.1 appear to contribute differing functions to cell survival in the myocardium (6, 7). Thus, it is important to understand the regulation of these two genes independently. We previously reported that NF-κB inhibition suppresses, but does not completely prevent, the IPC-induced increase of Hsp70.3 mRNA levels in the myocardium (6). Here we show that the late IPC-induced increase of Hsp70.3 protein expression is subject to post-transcriptional regulation after late IPC. Given the known importance of the 3'-UTR in post-transcriptional regulatory processes, this region of the Hsp70.3 gene was examined with regard to potential post-transcriptional regulatory elements. In addition to the predicted binding sites of 12 different miRNAs, the 3'-UTR of the Hsp70.3 mRNA sequence was predicted to harbor four distinct strong polyadenylation signals. Although generally attributed as an independent phenomenon, a prominent role for both miRNAs and alternative polyadenylation (APA) in post-transcriptional regulation of gene expression is now widely recognized (8, 9). Thus, we hypothesized that coordinated actions by miRNAs and APA exert post-transcriptional regulation on Hsp70.3 expression in the myocardium.

The results reported here show that IPC induces a decrease in the levels of the Hsp70.3-targeting miRNAs miR-378* and miR-711, both of which were found to exert post-transcriptional suppression on Hsp70.3 in vitro via the 3'-UTR. In addition, the results demonstrate the alternative polyadenylation of Hsp70.3 transcripts in the myocardium resulting in mRNA transcripts with varying 3'-UTR lengths. There is a significant and preferential increase in the levels of Hsp70.3 mRNA transcripts with shortened 3'-UTRs following Hsp70.3-inducing stimuli such as HS, IPC, or I/R. This APA-mediated shortening
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RNA Isolation and qRT-PCR—Left ventricular tissue was collected 3 h following an IPC stimulus as described above. For measurements of miRNA, total RNA was isolated using an acid phenol:chloroform technique and enriched for the small RNA fraction per the manufacturer’s instructions (mirVana miRNA isolation kit; Ambion). cDNA was synthesized from the small RNA-enriched fraction using an RT² first strand cDNA synthesis kit (SA Biosciences). Specific miRNA levels were assessed through quantitative real-time-PCR using miRNA-specific primers (SA Biosciences). For assessment of Hsp70.3 transcript levels, total RNA was isolated (RNaseq kit; Qiagen), and cDNA was synthesized (high capacity RNA-to-cDNA kit; Applied Biosystems) per the manufacturer’s instructions. All samples were performed in triplicate with a minimum of three independent experimental replicates with expression differences calculated using the ∆∆ Ct, approximation method using 18S mRNA as a loading control (14). Corrections for primer efficiency were made where appropriate using the Pfaffl method (15).

3’-RACE and Quantitative Assessment of APA—Qualitative 3’-RACE assessment of the Hsp70.3 3’-UTR population was performed via a PCR reaction with an Hsp70.3-specific primer and a modified oligo(dt)18 primer 5’-NT18-3’, with “N” representing A, G, or C, to allow for more specific binding at the beginning of the poly(A) tail and accurate size assessment of the 3’-UTR sequence. Quantitative measurements of the ratios of 3’-UTR transcripts were done via qRT-PCR using primers specific to sequence regions just proximal to the poly(A) site product being measured. To take into account that these primers would recognize polyadenylation products from any distal poly(A) site (i.e. primers recognizing sequence just proximal to poly(A) site 2 would detect product resulting from polyadenylation at site 2, 3, or 4), the quantitative result from each primer set is expressed as a normalized ratio to the total transcript as measured by primers just distal to the end of the protein coding sequence (proximal to all potential poly(A) sites). The resulting PCR products from both RACE and qRT-PCR were sequence-validated to be the proper Hsp70.3 3’-UTR products.

Cell Culture and Transfections—H9c2 cells were obtained from ATCC and grown under conditions of 5% CO₂, 95% air at 37°C in DMEM supplemented with 10% FBS. Wild-type and Hsp70.1 murine embryonic fibroblasts (MEFs) were isolated from 18-day-old embryos as described previously (16). HSF-1−/− and p65−/− MEFs were kind gifts from Drs. Hector Wong (Cincinnati Children’s Hospital) and Denis Guttridge (Ohio State University), respectively. Experiments were performed in either 96-well (reporter assays) or 6-well (Western blots) plates with an initial seed density of 3 x 10⁴ and 1 x 10⁴ cells/well, respectively; cells were transfected 24 h after initial seeding. For reporter assays, 30 ng of plasmid DNA/well was transfected using Lipofectamine 2000 (Invitrogen) at the recommended ratio of 2.5 μl/1.0 μg of plasmid DNA. RNAi (miRNA, siRNA, or negative control RNAi; Qiagen) was transfected at the indicated molar doses using Lipofectamine 2000 at the recommended ratio of 1.0 μl/20 pmol of RNAi. All transfections were done in reduced serum Opti-MEM, an equal volume of DMEM supplemented with 20% FBS was added 4 h following transfection, and cells were then cultured overnight prior to experimental treatment. Heat shock was accomplished by placing cells at
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RESULTS

Hsp70.3 Expression Is Subject to Post-transcriptional Regulation in the Myocardium—We previously showed that Hsp70.3 mRNA expression was induced 26.5-fold in the myocardium after late IPC in wild-type mice and 13.8-fold in 2M NF-κB dominant-negative mice (6). Assessment of total Hsp70 protein levels after late IPC showed a robust 7.7- and 14.3-fold increase in cytoplasmic and nuclear fractions, respectively, of Hsp70 protein expression in wild-type mice (Fig. 1). However, despite only a partial reduction in Hsp70.3 mRNA levels (26.5–13.8-fold) in 2M IPC relative WT IPC, no significant increase in total cytoplasmic or nuclear Hsp70 protein expression was observed in 2M NF-κB dominant-negative mice, relative to sham controls following IPC (Fig. 1). This result indicates that Hsp70 protein expression is subject to post-transcriptional suppression in 2M mice (with dominant-negative suppression of NF-κB activation) after late IPC in the heart.

Post-transcriptional Regulation of Hsp70.3 Is Mediated by the 3'-UTR—Post-transcriptional regulation of gene expression has most commonly been observed to be mediated through the 3'-UTR of the mRNA transcript (8, 9). Thus, luciferase reporter plasmids containing the 3'-UTR (~1.25 kb from the end of the protein coding sequence) of the Hsp70.3 gene were used to examine the regulatory role of the Hsp70.3 3'-UTR on expression. Inclusion of the Hsp70.3 3'-UTR reduced reporter expression by ~50% at basal levels as well as after HS in both MEF and H9c2 cells (Fig. 2). The suppressive effect of the Hsp70.3 3'-UTR sequence was observed with both a CMV promoter (Fig. 2) and the Hsp70.3 promoter, indicating that this suppressive effect is independent of promoter regulation and specific to the 3'-UTR sequence.

Late IPC Reduces the Expression of the Hsp70.3-targeting miRNAs—Analysis of the Hsp70.3 3'-UTR sequence with regard to miRNA binding sites revealed that 12 miRNAs are predicted to bind within the 3'-UTR (as determined via the Sanger miRBase target database) (Table 1) (17). Quantitative RT-PCR assessment of the myocardial expression levels of the 12 Hsp70.3-targeting miRNAs found that IPC induced a suppression of two Hsp70.3-targeting miRNAs: miR-378* and

| Number | miRNA ID | WT IPC vs. WT sham | NF-κB DN vs. WT IPC | NF-κB vs. WT sham |
|--------|----------|---------------------|----------------------|------------------|
| 1      | mmu-miR-485* | 1.95                | 1.29                | 2.51             |
| 2      | mmu-miR-224  | ~1.03               | ~1.45               | ~1.49            |
| 3      | mmu-miR-711  | ~2.63               | ~3.76               | ~1.43            |
| 4      | mmu-miR-7a*  | 1.13                | 1.25                | 1.41             |
| 5      | mmu-miR-139–5p | ~1.16             | ~1.63               | ~1.89            |
| 6      | mmu-miR-378* | ~1.75               | ~1.06               | ~1.85            |
| 7      | mmu-miR-342–3p | ~1.2              | ~1.36               | ~1.62            |
| 8      | mmu-miR-28   | ~1.09               | ~1.07               | ~1.16            |
| 9      | mmu-miR-301b  | 1.04                | 1.06                | 1.1              |
| 10     | mmu-miR-212  | ~1.25               | ~1.24               | ~1.55            |
| 11     | mmu-miR-490  | ~1.11               | ~1.44               | ~1.6             |
| 12     | mmu-miR-301a | 1.01                | ~1.16               | ~1.16            |
miR-711. Post-IPC, levels of miR-711 were found to be reduced in WT mice, but not 2M mice, indicating that post-IPC suppression of miR-711 is dependent on NF-κB activation in the cardiac myocyte (Table 1). IPC-induced suppression of miR-378* levels was independent of NF-κB (Table 1).

miR-378* and miR-711 Mediate Post-transcriptional Suppression of Hsp70.3 Expression via the 3'-UTR—Next, the ability of exogenous miR-378* or miR-711 to inhibit expression of the Hsp70.3 3'-UTR luciferase reporters was examined in H9c2 rat cardiac myoblast cells. To test miR-378* and miR-711-mediated regulation of the Hsp70.3 3'-UTR, H9c2 cells were transfected with CMV-luciferase-70.3 3'-UTR reporter (CMV-Luc-U3) and increasing doses of miR-378*, miR-711, a non-targeting negative control RNAi, or an Hsp70.3-targeting siRNA (Fig. 3 and supplemental Fig. S1). Results show that treatment with miR-378*, miR-711, or an Hsp70.3-targeting siRNA led to a dose-dependent reduction in CMV-Luc-U3 expression when compared with treatment with an equal dose of non-targeting negative control siRNA (Fig. 3A). The miR-378* and miR-711-mediated suppression of the CMV-Luc-U3 reporter is sequence-specific to the Hsp70.3 3'-UTR as evidenced by the inability of the miRNAs to reduce expression of a similar reporter lacking the Hsp70.3 3'-UTR sequence (Fig. 3B).

The effects of miR-378* and miR-711 on endogenous protein levels were also examined using Hsp70.1 knock-out MEFs to allow for easy measurement of Hsp70.3 protein levels without interference from Hsp70.1; an antibody able to distinguish between the inducible Hsp70 genes is not available. Results show that treatment with either 100 nM miR-711 or 100 nM miR-378* is able to reduce basal expression of Hsp70.3 protein, as measured by Western blotting (Fig. 4A). However, treatment with miR-711, but not miR-378*, is able to reduce Hsp70.3 protein induction following a 1-h heat shock treatment (Fig. 4B).

The Hsp70.3 mRNA Transcript Is Expressed with Multiple Distinct 3'-UTR Lengths as a Result of Alternatively Polyadenylated Transcripts—In addition to miRNA binding sites, the Hsp70.3 3'-UTR sequence is also predicted to contain four distinct polyadenylation recognition sites (Fig. 5). 3'-RACE was used to survey for the expression of Hsp70.3 transcripts harboring 3'-UTRs of different sizes as a result of APA. Results using either primary MEF cells or in vivo myocardium show the presence of multiple distinct size populations of Hsp70.3 3'-UTR lengths (Fig. 6). The arrows in the figure depict predicted PCR fragment sizes correlating with APA at each of the four predicted polyadenylation sites. These qualitative RACE results suggest that Hsp70.3 polyadenylation occurs primarily at APA sites 2 and 3. Interestingly, the annotated GenBankTM sequence
for Hsp70.3 does not indicate expression of the 3′/H11032-UTR past APA site 2. However, a more detailed examination of expressed sequence tags located at least one expressed sequence tag (CO041885.1) that does represent a long variant of the Hsp70.3 3′-UTR supporting the use of APA site 3.

Induction of Hsp70.3 mRNA Expression Results in Shortening of the Hsp70.3 3′-UTR—Hsp70.3 mRNA levels were assessed in WT MEF cells following a 1-h HS using qRT-PCR designed to assess the expression level of each Hsp70.3 APA product through amplification of specific regions of the Hsp70.3 transcript relative to the four polyadenylation sites (APA 1–4) (Fig. 7A). Results show that both total Hsp70.3 mRNA transcript and total APA 2 mRNA transcript increase to the same levels and that both peak at 2 h following the onset of HS, indicating that there is no significant truncation or polyadenylation of the mRNA transcript at APA site 1 (Fig. 7B). APA 3 transcript, however, peaks at 1 h following HS, is not up-regulated to the same degree, and is reduced back to baseline more quickly than total or APA 2 transcript (Fig. 7B). In addition, as total post-HS expression of total Hsp70.3 mRNA increases, the ratio of APA 3:total transcript decreases, indicating that the 3′-UTR of the transcript is shortened via APA at site 2 rather than at site 3 (Fig. 7C).

Negligible expression of the Hsp70.3 mRNA region distal to poly(A) site 3 (APA 4 transcript) was detected, indicating that the vast majority of Hsp70.3 transcripts are polyadenylated at either APA site 2 or APA site 3. This lack of a detected signal from polyadenylation at APA site 4 is supported by predictions showing APA site 4 to be the weakest of the four predicted APA sites (DNA Functional Site Miner).

To confirm a physiological role of APA on expression of Hsp70.3, we compared expression from luciferase reporters with either the full-length Hsp70.3 3′/H11032-UTR or a version truncated just past APA site 2 to mimic the APA-induced shortening observed in the endogenous gene. As previously observed in Fig. 2, inclusion of the full-length 3′/H11032-UTR results in a significant suppression of reporter expression, but when the shortened 3′-UTR sequence truncated just past the APA 2 was included, this suppressive effect was no longer observed (Fig. 7D). This result supports the hypothesis that APA-mediated truncation of this sequence from the 3′-UTR acts to enhance expression of the gene.

APA-mediated Truncation of the Hsp70.3 3′-UTR Relieves Suppression through Both Enhanced mRNA Stability and Translational Efficiency—To determine whether the suppressive effect of the 3′-UTR is a post-transcriptional effect exerted directly on the mRNA transcript or a silencing of translation, the levels of expressed mRNA from reporter genes with and without the Hsp70.3 3′-UTR were assessed. First, reporters driven by the endogenous Hsp70.3 promoter were used such
that transcriptional induction could be triggered by an HS stimulus. Results show that a 1-h HS triggers an immediate and robust increase in reporter transcripts regardless of the presence of the Hsp70.3 3′-UTR sequence. However, the reporter including the Hsp70.3 3′/H11032-UTR shows a much quicker decline from peak levels when compared with a similar reporter lacking the Hsp70.3 3′-UTR (Fig. 8A). This result is in agreement with results from Fig. 7 showing that levels of the endogenous transcript containing the full-length 3′-UTR return to baseline before levels of the APA-shortened transcript and suggests that shortening of the Hsp70.3 3′-UTR may enhance the stability of the mRNA transcript.

Next, the mRNA and protein levels were simultaneously measured from a CMV-luciferase reporter with and without the Hsp70.3 3′-UTR. Unlike the Hsp70.3 promoter-driven reporter, the transcriptional expression of which is acutely stimulated and reduced after a 1-h HS, the CMV promoter-controlled reporters drive a continuously high level of transcriptional expression. We could not detect significant 3′-UTR-dependent differences in mRNA levels from CMV promoter-controlled reporters for the first 8 h following transfection (Fig. 8B). Although a significant difference in mRNA levels is apparent at 12 h after treatment, a significant suppression of protein levels from the reporter with the Hsp70.3 3′-UTR is observed as early as 4 h after treatment and persists throughout the assessed time points (Fig. 8, B and C). The difficulty in detecting differences in mRNA levels related to the presence of the 3′-UTR likely reflects the fact that the CMV promoter drives strong constitutive expression of mRNA. We conclude that, in addition to altering the stability of the mRNA, it appears that the removal of the Hsp70.3 3′/H11032-UTR sequence increases protein levels.

Hsp70.3 APA-mediated 3′-UTR Shortening Is Observed in the Heart in Vivo following IPC or I/R—Total Hsp70.3 transcript levels in the in vivo myocardium are increased in a partially NF-κB-dependent manner following IPC or I/R (Figs. 1 and 9A) (6). Similar to what was observed following HS-induced Hsp70.3 mRNA expression, in both IPC and I/R, the increase in total Hsp70 mRNA is accompanied by an APA-mediated shortening of the Hsp70.3 3′-UTR (Fig. 9B). To investigate the mechanism of Hsp70.3 APA, the role of NF-κB in this process was investigated using 2M 1xBα dominant-negative mice. This mouse model of transgenic 1xBα dominant-negative inhibition of NF-κB was used because it was in these mice following IPC that the post-transcriptional regulation of Hsp70.3 was first observed (Fig. 1). However, similar to what was observed in the
wild-type mice, results show a significant reduction in the ratio of APA product 3 in the 2M mice following both IPC and I/R (Fig. 9B). Thus, upon a stimulus that induces Hsp70.3 mRNA expression, the Hsp70.3 3′-UTR is expressed as a shortened form due to increased APA at polyadenylation site 2. The APA effect was also observed to be unaltered in MEF cells deficient for the p65 (RelA) subunit of NF-κB (Fig. 9C). Taken together, these results indicate that NF-κB activity does not play a regulatory role in alternative polyadenylation of the Hsp70.3 3′-UTR.

The Transcription Factor HSF-1 Is Needed for Alternative Polyadenylation of the Hsp70.3 3′-UTR—Because the transcriptional regulator of heat shock, HSF-1, has been previously suggested to play a role in mRNA polyadenylation (18), we also examined the role of HSF-1 in HS-mediated APA of the Hsp70.3 transcript. Results using HSF-1−/− MEFs (the homozygous mice are not viable) demonstrate that the Hsp70.3 3′-UTR does not undergo an APA-mediated shortening following HS in MEF cells lacking HSF-1 (Fig. 9C). These results implicate an obligatory role for the transcription factor HSF-1 in APA-mediated shortening of the Hsp70.3 3′-UTR.

**DISCUSSION**

The results presented here demonstrate that Hsp70 protein expression in the myocardium is subject to post-transcriptional regulation after late IPC. We show that in addition to NF-κB-dependent transcriptional up-regulation of the gene (6), late IPC functions to remove microRNA inhibition via (i) NF-κB-dependent reduction of miR-711 (Hsp70.3-suppressive) levels, (ii) NF-κB-independent reduction of miR-378* (Hsp70.3-suppressive), and (iii) HSF-1-dependent APA, which acts to remove the suppressive miR-378* binding site from the
Hsp70.3 3'-UTR. We further provide evidence that the suppressive effects of the miRNAs result in reduction in Hsp70.3 mRNA levels and a decrease in Hsp70.3 protein. Specifically, it appears that the shortening of the 3'-UTR by APA enhances the amount of protein produced, perhaps by increased translation efficiency.

The role of miRNAs in modulating gene expression in response to ischemic stimuli in the heart is coming to light as more and more gene products are being functionally identified as targets of miRNA regulation (19). A direct regulation of Hsp70 expression by miRNAs in the heart has not yet been demonstrated. However, in a recent study by Yin et al. (20), it
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was shown that IPC increases the expression of the miRNAs miR-23b, miR-483, and miR-1 in the heart and that administration of these miRNAs was sufficient to protect the myocardium from subsequent I/R injury. Although this effect was suggested to be due to an miRNA-mediated increase in endothelial NOS (eNOS), HSF-1, and Hsp70 expression, this is likely to be an indirect effect on Hsp70 expression due to the fact that there are no predicted target sites for these miRNAs in the Hsp70.3 gene sequence (20).

Our previous work showed that NF-κB activation in cardiac myocytes is necessary for Hsp70.3 expression in the heart following IPC and I/R (6). However, NF-κB does not appear to play a role in the alternative polyadenylation of the Hsp70.3 mRNA transcript. The lack of increased Hsp70.3 protein expression following IPC in 2M mice despite only a partial decrease in mRNA levels (Fig. 1) and an NF-κB-independent APA-induced shortening of the 3′-UTR (Fig. 9, B and C) suggests that other processes in addition to APA shortening of the 3′-UTR are involved in post-transcriptional regulation of Hsp70.3 expression. NF-κB-dependent down-regulation of miR-711 levels, as observed in IPC (Table 1), likely represents one of these additional processes. The results presented here are novel in that they demonstrate a stimulus-specific regulation of alternative polyadenylation that has direct consequences on the miRNA-mediated post-transcriptional regulation. Specifically, the removal of the miR-378* binding site in the truncated Hsp70.3 APA product removes the action of miR-378*, rendering regulation of the shorter transcripts relatively more dependent upon NF-κB-dependent changes in miR-711.

Alternative polyadenylation has been previously shown to be a potentially critical post-transcriptional regulator of gene expression (9, 21). The primary means by which this occurs is presumed to be through the removal of regulatory sequence elements within the 3′-UTR of the mRNA transcript, thus affecting miRNA binding, mRNA stability, or binding of other proteins or regulatory factors, including those involved in translation. The loss of regulatory elements in the 3′-UTR due to APA-mediated shortening of the transcript has recently been recognized as means by which oncogenes may escape regulation in cancer cells (21). Similarly, our results show that increased expression of Hsp70.3 mRNA in the myocardium is correlated with a shortening of the 3′-UTR that results in the loss of a predicted binding site for a suppressive miRNA. Experiments with acute 1-h heat shock induction (Fig. 8A) demonstrate that although the induction kinetics and peak mRNA levels are similar, the decrease in mRNA levels after 3 h is faster for the luciferase reporter mRNA that contains the Hsp70.3 3′-UTR. Taken together with data that the Hsp70.3 mRNA with the APA 2 terminus decreases in level more quickly after heat shock than the APA 3 transcript, this supports a reduced stability of the mRNA with the APA 2 termination. In addition, our results suggest that the APA-shortened version of the Hsp70.3 mRNA results in more efficient protein translation than its full-length counterpart (Fig. 8). Note that because these studies were performed with the luciferase reporter, differences in Hsp70.3 protein stability would not play a role in this effect. Other studies have also associated alternative polyadenylation with direct effects on translation efficiency and protein expression. Brain-derived neurotrophic factor (BDNF), chloramphenicol acetyltransferase (CAT), and the apoptosis gene hap have been shown to be expressed as multiple alternative polyadenylation products, with one product displaying a much higher efficiency of protein expression (22–24).

Although as many as 50% of expressed genes have been identified to contain alternative polyadenylation sites (25), the role of alternative polyadenylation in the regulation of gene expression remains an underexplored field. In addition to Hsp70.3, COX-2 is a well known NF-κB-dependent cardioprotective gene product that has been shown to be subject to alternative polyadenylation (26–29). In the case of COX-2 alternative polyadenylation, it has been shown that this process is dependent upon the interaction of a discrete set of RNA-binding proteins (polyrymidrine tract binding protein (PTB), PTB associated splicing factor (PSF), p54 non-related binding protein (p54NR), and U1A) with upstream sequence elements in the 3′-UTR that control the efficiency of polyadenylation (29). In addition to the core polyadenylation factors such as cleavage/polyadenylation specificity factor (CPSF), cleavage stimulation factor (CstF), cleavage factor I (CFI), and cleavage factor II (CFII), additional factors including transcriptional and splicing factors whose specific role in polyadenylation is not yet completely understood have also been identified as mediators of the polyadenylation process (9). Whether or not these or similar RNA-binding proteins play a role in the regulation of Hsp70.3 APA remains to be seen. It is still not entirely clear how the activity of many of these proteins or their modulation of polyadenylation specificity or efficiency is controlled within the cell.

Our results suggest that in the absence of HSF-1, APA of the Hsp70.3 gene is abrogated (Fig. 9C). However, it is unclear from these data whether this is a direct or an indirect role for HSF-1 in APA of Hsp70.3. The notion that a transcription factor may play a regulatory role in polyadenylation is not a new one as it is has been previously shown that mRNA processing is linked to transcription and that polyadenylation factors can directly interact with transcription factors (30, 31). Previous work suggests that HSF-1 can physically interact with the polyadenylation factors symplekin and CstF64, potentially playing a role in polyadenylation of the Hsp70 genes (18). However, at this point, the identity of the regulatory factors controlling polyadenylation site selection within the Hsp70.3 mRNA remains unknown and is the subject of ongoing/future work.

In addition to the identity of the RNA-binding proteins responsible for alternative polyadenylation of the Hsp70.3 transcript, there are other unanswered questions. First, is APA-mediated shortening of the Hsp70.3 3′-UTR directly linked to the same stimuli that lead to a transcriptional induction of the Hsp70.3 gene, or is there differential APA regulation? Second, do miRNAs or other small non-coding RNAs play direct roles in the regulation of alternative polyadenylation? Finally, although we show here how APA plays a direct role in regulating the binding of miR-378*, by removing its binding site, the question of whether miRs or other non-coding RNAs play critical roles in regulating APA remains unanswered. It is possible that co-evolution of the miRNA and APA regulatory processes has resulted in some interesting regulatory interactions in this regard.
In conclusion, our results indicate that alternative polyadenylation plays a critical role as one of multiple integrated regulatory steps controlling Hsp70.3 gene product expression. At basal levels, constitutive expression levels of the mRNA and protein are both readily detectable. Upon an Hsp70.3-inducing stimulus, the protein up-regulation is achieved through the coordinated actions of: 1) transcriptional induction of the gene (partially dependent on NF-κB); 2) decreased expression levels of Hsp70.3-targeting miRNAs (as measured in the in vivo myocardium after late IPC; miR-711 was decreased in an NF-κB-dependent manner, and miR-378* was NF-κB-independent); and 3) alternative polyadenylation-mediated shortening of the 3′-UTR resulting in loss of a suppressive miRNA binding site, mRNA stabilization, and increased protein production. It is interesting that in addition to their roles in transcriptional modulation, NF-κB and HSF-1 both play coordinated roles in post-transcriptional regulation of Hsp70.3: NF-κB through regulation of the Hsp70.3-targeting miR-711 and HSF-1 through regulation of APA. These results enhance the understanding of how miRNAs and alternative polyadenylation contribute to the post-transcriptional regulation of Hsp70.3 and may be extended to additional genes whose expression is subject to similar means of post-transcriptional control through microRNAs and alternative polyadenylation.

Given the emerging role for APA as an important post-transcriptional regulator of gene expression, the significance of these results has implications that stretch far beyond just the understanding of Hsp70.3 gene expression in myocardium. APA represents an underexplored, yet critical, regulatory step in the control of gene expression. The increased mechanistic understanding of APA and its integration with miRNA regulatory networks will greatly increase our knowledge of post-transcriptional gene regulation and may lead to the identification of novel therapeutic targets for the treatment of diverse pathologies such as cancer, diabetes, neurodegenerative diseases, and aging in addition to post-myocardial infarction pathophysiology and cardiomyopathy in the cardiovascular system.

Acknowledgments—We thank Jackie Belew for support in maintaining mouse breeding colonies and organizing procedures and for research assistance and Hector Wong (Cincinnati Children’s Hospital) and Denis Guttridge (Ohio State University) for providing us with HSF-1−/− and p65−/− MEF cells, respectively.

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