Regulation of Ribosomal DNA Transcription during Contraction-induced Hypertrophy of Neonatal Cardiomyocytes*

(Received for publication, July 26, 1995, and in revised form, October 19, 1995)

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Cardiac hypertrophy requires protein accumulation. This results largely from an increased capacity for protein synthesis, which in turn is the result of an elevated rate of ribosomal biogenesis. The process of ribosome formation is regulated at the level of transcription of the ribosomal RNA genes. In this study, we examined the amounts and activities of various components of the ribosomal DNA transcription apparatus in contraction-arrested neonatal cardiomyocytes and in spontaneously contracting cardiomyocytes that hypertrophy. Nuclear run-on assays demonstrated that spontaneously contracting cardiomyocytes supported a 2-fold increased rate of ribosomal DNA transcription. However, enzymatic assay of total solubilized RNA polymerase I and Western blots demonstrated that contraction-induced increases in ribosomal RNA synthesis were not accompanied by increased activity or amounts of RNA polymerase I. In contrast, accelerated ribosome biogenesis was accompanied by an increased amount of the ribosomal DNA transcription factor, UBF. Immunoprecipitation of [\(^{32}P\)]orthophosphate-labeled UBF from hypertrophying, neonatal cardiomyocytes indicated that the accumulated UBF protein was phosphorylated and, thus, in the active form. UBF mRNA levels began to increase within 3–6 h of the initiation of contraction and preceded the elevation in rDNA transcription. Nuclear run-on assays demonstrated increased rates of transcription of the UBF gene. Transfection of chimeric reporter constructs containing deletions of the 5′-flanking region of the UBF gene revealed the presence of contraction response elements between −1189 and −665 relative to the putative start of transcription. These results are consistent with the hypothesis that UBF is an important factor in the regulation of rDNA transcription during contraction-mediated neonatal cardiomyocyte hypertrophy.

Mechanical stimulation, in its various forms, is a primary determinant of cardiomyocyte phenotype and hypertrophic growth both in vivo and in vitro (reviewed in Refs. 1 and 2). For instance, unattached feline cardiomyocytes maintained in cell suspension rapidly lose the organizational characteristics of differentiated cardiac muscle and resemble undifferentiated neonatal cardiomyocytes (3). In contrast, attachment of adult cardiomyocytes to laminin-coated substrates inhibits the loss of characteristic biochemical and functional properties (3). In recent reports, linear deformation (passive stretch) has been demonstrated to increase cellular growth as measured by rates of RNA and protein synthesis and protein accumulation in cultured neonatal cardiomyocytes (4, 5).

Spontaneous contractile activity also appears to be an important regulator of cardiomyocyte growth (6–8). For example, spontaneously contracting neonatal cardiomyocytes in culture accumulate RNA and protein at a faster rate than noncontracting, i.e. arrested, cells. This growth occurs in the absence of changes in DNA content or cell number (hypertrophic growth) and appears to be largely independent of humoral or neuronal factors (8). Increased protein levels are due to an increase in the fractional rate of protein synthesis in the absence of changes in the rate constants of protein degradation. The elevated RNA content represents, for the most part, an accrual of ribosomes, i.e. ribosomal RNA (rRNA), which is necessary to support the elevated cellular capacity to produce protein (9). However, little, if anything, is known about the signals which couple contraction to ribosome biogenesis and subsequent protein synthesis.

In other mammalian cell types and other classes of organisms, ribosome content is regulated largely by alterations in the rate of transcription of the RNA genes (rDNA) to produce 45 S preribosomal RNA, rather than by changes in the processing or stability of ribosomal RNA (rRNA) (10, 11). Similarly, the accelerated levels of ribosome biogenesis observed during contraction-induced hypertrophy of cultured neonatal cardiomyocytes is linked to elevated rates of rRNA synthesis (12). Increased rRNA transcription also accounted for the accelerated rates of ribosome biogenesis that follow exposure to norepinephrine (13), phorbol 12-myristate 13-acetate (14), or endothelin 1. Overall, these results emphasize that rRNA transcription is a central point in the control of cardiomyocyte protein synthesis during cardiac growth.

Efficient transcription of ribosomal DNA promoters in vitro requires at least two DNA-binding proteins, UBF and SL-1, as well as RNA polymerase I (10, 11, 15). In addition, recognition by RNA polymerase I of the protein-DNA complexes that form on the promoter requires the presence or activity of at least one polymerase-associated factor referred to as either TF1C or TIF-1A (16, 17). SL-1 is absolutely required for basal levels of transcription in vitro (10, 11), and UBF increases the efficiency of template utilization raising the level of transcription (10, 11, 18). When fractionated by SDS-PAGE, mammalian UBF consists of two proteins, 97 and 94 kDa, that are referred to as UBF1 and UBF2, respectively (10, 11, 20). They are coded for by two different mRNAs that result from alternative processing.

* This work was supported in part by National Institutes of Health Grants HL47638 and GM48991 (to L. I. R.) and an award from the Geisinger Foundation (to L. I. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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2 The abbreviations used are PAGE, polyacrylamide gel electrophoresis; CAT, chloramphenicol acetyltransferase; rPolI, r subunit of RNA polymerase I.
of a single transcript (17). UBF1 and UBF2 bind to DNA and form homo- and heterodimers, but only UBF1 has been shown to activate transcription in vitro (19, 21, 22).

The activity of UBF is regulated by post-translational and/or transcriptional mechanisms (23–26). In regard to the former mechanism, UBF is a phosphoprotein whose ability to activate transcription is reduced when dephosphorylated (23, 24). When CHO cells were serum-starved, and rDNA transcription significantly reduced, the phosphorylation state of UBF also decreased (23). In contrast, the decreased rDNA transcription observed during serum starvation of NIH3T3 fibroblasts was characterized by decreased UBF content. In this example, the decrease in UBF protein was preceded by a reduction in UBF mRNA content (26).

We have examined the rDNA transcription apparatus of contraction-induced hypertrophic cardiomyocytes in order to determine which components of this system are altered in response to the hypertrophic stimulus. We show here that the accelerated rate of rDNA transcription during contraction-mediated neonatal cardiomyocyte hypertrophy is not accompanied by increased amounts or activity of RNA polymerase I enzyme, but by a significant increase in the protein and mRNA levels of UBF. Nuclear run-on assays of UBF mRNA synthesis revealed that at least part of the increase in UBF content was the result of increased transcription of the UBF gene. Transient transfection of chloramphenicol acetyltransferase (CAT) reporter constructs linked to fragments 5′ of the translation initiation site of the UBF gene demonstrated the presence of contraction response elements between −1189 and −665 relative to the start of transcription (+1).

MATERIALS AND METHODS

Cardiomyocyte Culture—Neonatal cardiomyocytes were isolated from the ventricles of day-old Sprague-Dawley rat pups as described previously and were maintained in defined serum-free media (27), containing 0.1 mm/liter bromodeoxyuridine, and maintained in the same defined media for the duration of the experiments. KCl (50 mmol/liter) was added to the medium to prevent the spontaneous contraction characteristic of neonatal cardiomyocytes plated at high density (6, 8, 9, 12). Cultures prepared in this manner consisted of 85–95% cardiomyocytes. For some experiments, the isolated cardiomyocytes were purified further by centrifugation through Percoll (Pharmacia Biotech Inc.) step gradients as described (28). We were unable to detect fibroblasts in these cultures as determined by immunocytochemical staining with an anti-fibroblast actin antibody (39).

Induction of Cardiomyocyte Hypertrophy—After 2 days in defined media (day 3 of culture), spontaneous contraction of the cardiomyocytes was reinitiated by reducing the level of KCl in the media from 50 mm to 5 mm. Spontaneous contraction occurred within 1–2 h of the media change. Control, contractile arrested cells were maintained in media containing 50 mm KCl. After the appropriate length of treatment, cells were harvested and processed for the determination of protein and DNA, or for Northern, Western, or nuclear run-on assays. Protein and DNA Determination—Cardiomyocytes were solubilized directly in 1× SSC (0.15 mol/liter NaCl, 0.015 mol/liter sodium citrate), 0.25% sodium dodecyl sulfate and frozen at −20°C until analysis. DNA determinations were performed by the fluorometric method of Cesareno et al. (29) using calf thymus DNA as a standard. Protein was assayed using the Bio-Rad DC assay kit with immunoglobulin G (IgG) as the protein standard. Growth was expressed as the percentage increase in the protein to DNA ratio of spontaneously contracting cells over time as compared to contractile-arrested (control) cells. Experiments were repeated a minimum of 5 times from separate cardiomyocyte preparations.

Determination of Total Solubilized RNA Polymerase I Activity—RNA Polymerase I activity was measured in solubilized preparations derived from arrested and contracting neonatal cardiomyocytes as described previously (30, 31). Briefly, 80 × 10⁶ cells were resuspended in 3 volumes of TGED buffer (50 mm Tris-HCl, pH 7.9, 25% glycerol, 0.1 mm EDTA, 0.5 mm dithiothreitol, 0.5 mm phenylmethylsulfonyl fluoride), the salt concentration was adjusted to 0.32–0.34 M with (NH₄)₂SO₄, and the suspension was sonicated four times while cooling in an ice slurry. The sonicates were overlaid with mineral oil and centrifuged at 67,000 × g for 90 min. The salt concentration of the supernatant was adjusted to 100 mm (NH₄)₂SO₄ by the addition of TGED, and the suspension was centrifuged at 67,000 × g for 90 min. This supernatant was diluted to a final salt concentration of 50 mm (NH₄)₂SO₄ and again centrifuged for 90 min at 67,000 × g. The supernatant obtained from this third centrifugation (F3) was frozen in aliquots at −80°C. The polymerase activity was measured by incubating 50 μl of F3 with 50 μl of substrate mixture (100 mm Tris-HCl, pH 7.9, 5 mm β-mercaptoethanol, 1 mm ATP, 1 mm CTP, 1 mm GTP, 16 μM UTP, 2.0 μCi of [3H]UTP (35 Ci/mmol), 3.4 mm MgCl₂, and 0.5 mg/ml calf thymus DNA) at 37°C for 15 min. The incorporation of [3H]UMP in RNA was measured as described (30, 31). RNA polymerase II activity was measured by the DNA synthesis in the absence and presence of 5 μg/ml α-amanitin, and RNA polymerase I by the incorporation that took place in the presence of 200 μg/ml α-amanitin.

RNA Extraction and Northern Analysis—Total RNA was extracted from cardiomyocytes as described (32). Following electrophoresis through 1.2% denaturing (formaldehyde) agarose gels (33), the RNA (30 μg/lane) was blotted onto Zeta-Probe (Bio-Rad) membranes according to the manufacturer’s instructions. Detection and quantitation of UBF mRNA was carried out as described (13, 34).

Western Analysis—Cardiomyocytes were rinsed 3 times in ice-cold phosphate-buffered saline, then scraped directly into 1× SSC, 0.25% sodium deoxycholate, and frozen at −20°C until analysis. Protein determination was performed as described above, and Western blots with anti-UBF and anti-RNA polymerase I antibodies were carried out as described previously (13).

Nuclear Run-On Transcription—Transcription from the rDNA and RNA polymerase II promoters in isolated cardiomyocyte nuclei was measured by the hybridization of in vitro synthesized, [32P]P-labeled run-on transcripts to a 45 S rDNA clone (p515/EKX) or UBF rDNA clone (pBSR-UBF405), respectively, as described previously (26). Equivalent amounts of nuclei from control and contracting cardiomyocytes were assayed per time point. The hybridization conditions and the post-hybridization washes were the same as described (35). Hybridization was detected by autoradiography.

Phosphorylation Studies—Measurement of the phosphorylation status of UBF was carried out by immunoprecipitation of [32P]orthophosphate-labeled UBF protein from cardiomyocyte extracts using polyclonal anti-UBF antisera (23). Briefly, arrested neonatal cardiomyocytes were prelabeled with [32P]orthophosphate (1 mCi/60-mm dish) for 12 h after which the cells were then removed into fresh media containing 1 mCi/dish [32P]orthophosphate and either 50 mm KCl (arrested) or 5 mm KCl (contracting). After the appropriate time, the cells were washed twice in phosphate-buffered saline and scraped directly into 500 μl of modified RIPA buffer (36). UBF was immunopurified from the extracts by incubation overnight at 4°C with 50 μl of anti-UBF antibody coupled to 20 μl of protein A-agarose beads. After 4 × 1 ml of [10x modified RIPA buffer (36), the beads were boiled for 10 min in the presence of 2 × Laemmli solubilizing buffer. Phosphorylated UBF1 and UBF2 were resolved by SDS-PAGE and visualized by autoradiography.

Construction of Chimeric UBF Genes—Six fusion genes were used in this study (see Fig. 6). The genes were generating by polymerase chain reaction from the UBF gene using primers specific for the designated 5′ ends of each construct and a common 3′ primer (5′-TCGGTGGCCCT- GGCCGCGG-3′) that was complementary to nucleotides +78 to +93 of the UBF gene (+1 being the transcription initiation site) (37). The polymerase chain reaction fragments were inserted into the HindIII and Xbal sites of pCAT-Basic (Promega Corp.). The orientations of the inserts were confirmed by sequencing and restriction endonuclease mapping.

Transfection and CAT Assays—After 2 days in culture, contracting, neonatal cardiomyocytes, cultured as described above, were co-transfected with the indicated constructs (3 μg) and pCMV-βGal (0.5 μg) (Promega) using Lipofectamid (Life Technologies, Inc.). Five hours after transfection, the culture medium was replaced with fresh serum-free defined medium containing either 5 mm (beating) or 50 mm (arrested) KCl. Twenty-four hours later, the cells were harvested, and lysates were prepared as described (33) and frozen at −80°C until assayed for either chloramphenicol acetyltransferase (CAT) or β-galactosidase activity (33). The synthesis of acetylated chloramphenicol was measured by separating acetylated [14C]chloramphenicol from unmod-
**RESULTS**

Induction of Cardiac Hypertrophy and rDNA Transcription—Neonatal cardiomyocytes were cultured as described under "Materials and Methods" and maintained at a density of 4 x 10^6 cells/60-mm dish in media containing 50 mM KCl to arrest spontaneous contraction. Experiments were initiated after 3 days of culture to allow the cells to adapt to the culture conditions. During the subsequent 4 days in culture, there were no significant changes in protein/DNA ratios in contraction-arrested neonatal cardiomyocytes (Fig. 1A). These results are similar to those obtained by others using this system (8, 9).

To initiate hypertrophy, the cardiomyocytes were allowed to resume spontaneous contraction by reduction of the concentration of KCl in the culture medium from 50 mM to 5 mM. Within 2 h of the medium change, the cardiomyocytes were contracting, and, after 3 days, the contracting cardiomyocytes had accumulated 39% more protein than control, contraction-arrested cells (Fig. 1A). The increases in protein content of the cells occurred in the absence of changes in DNA content indicating that the growth was due to hypertrophy rather than hyperplasia. These results are in good agreement with those reported previously (8, 9).

In order to characterize the rates of ribosome biogenesis in our cultures, we used nuclear run-on analysis to measure the rate of ribosomal DNA (rDNA) transcription in contractile-arrested and spontaneously contracting neonatal cardiomyocytes. Results presented in Fig. 1B demonstrate that the rates of rDNA transcription in nuclei derived from spontaneously contracting neonatal cardiomyocytes were greater than those observed in nuclei obtained from time matched contractile-arrested cardiomyocytes. Significant increases in the rates of rRNA synthesis were observed within 12 h following initiation of contraction (159% ± 13) and reached maximal levels within 24–48 h (200% ± 21, Fig. 1B). These results confirm previous studies (8, 9, 12) indicating that contraction-induced hypertrophy of neonatal cardiomyocytes is associated with significant increases in the rate of synthesis of rRNA as the result of accelerated rates of transcription of the 45 S preribosomal DNA.

Analysis of the Cellular Content and Activity of RNA Polymerase I and UBF in Hypertrophic Cardiomyocytes—As an initial step in characterizing the regulation of rDNA transcription in neonatal cardiomyocytes, we measured the relative activity and content of RNA polymerase I and UBF in control and hypertrophic cardiomyocytes.

The relative enzymatic activity of total solubilized RNA polymerase I (as determined by its ability to initiate nonspecific transcription on calf thymus DNA) was determined in F3 extracts obtained from arrested and spontaneously contracting neonatal cardiomyocytes as described under "Materials and Methods." The activity of RNA polymerase I extracted from cardiomyocytes that had been contracting for 24 h was not significantly different from that extracted from contraction-arrested cells (106% ± 3.8, n = 3). Similarly, no significant differences were observed in the total polymerase (106% ± 5.8) or polymerase II (103% ± 16) activities in contracting cells with respect to contraction-arrested cells. Western analyses, using antibodies raised to the β subunit of RNA polymerase I (rPol β) (13), indicated that the levels of the β subunit of RNA polymerase I were essentially the same in contractile-arrested neonatal cardiomyocytes (Fig. 2A, lanes 1 and 3) and cells growing in response to spontaneous contraction (Fig. 2A, lanes 2 and 4). The results from a number of separate experiments were quantified by laser densitometry and are presented in Fig. 2B.

In many cell lines, the cellular content of UBF is proportional to the rate of rDNA transcription (25, 26). Accordingly, we determined whether the accelerated rates of rDNA transcription observed in spontaneously contracting neonatal cardiomyocytes might also be characterized by changes in the levels of UBF. As shown in Fig. 3A, UBF1 and UBF2 protein levels were significantly greater in rapidly contracting cardiomyocytes than in time-matched contractile-arrested cells. The UBF protein levels were maximal after 12 h of contraction (3.5–4.5-fold) and remained at similar levels for up to 72 h. The increases in UBF protein levels cannot be explained by the

![Fig. 1. Contraction regulates protein accumulation and rDNA transcription in neonatal cardiomyocytes.](image-url)
general increase in cellular protein observed in response to contraction, because equal amounts of protein were loaded per lane. The results from a number of separate experiments were quantified by laser densitometry and are presented in Fig. 3B.

Immunoprecipitation of Phosphorylated UBF from Neonatal Cardiomyocytes—Since the degree of UBF phosphorylation affects its ability to activate transcription in cell-free extracts (22, 23), we examined whether the phosphorylation status of UBF may differ between contractile-arrested and spontaneously contracting neonatal cardiomyocytes. The results in Fig. 3C demonstrate that a 4-5-fold increase in [32P]orthophosphate-labeled UBF was immunoprecipitated from cardiomyocytes which had been contracting for 12 h relative to the radioactivity recovered from an equal number of arrested cardiomyocytes (Fig. 3C, lanes 3 and 4, Autorad.). The increase in UBF phosphorylation was not merely part of a global increase in cellular protein phosphorylation in response to contraction since the total radioactivity of [32P]-labeled proteins in the supernatants of arrested and contracting cardiomyocytes were the same (Fig. 3C, lanes 1 and 2, Autorad.). Western analysis of the same immunoprecipitation samples fractionated on a parallel gel demonstrated a similar 4-5-fold increase in total UBF protein immunoprecipitated from contracting cardiomyocytes relative to contraction-arrested cells (Fig. 3C, lanes 3 and 4, Western). At the same time, UBF protein was not detectable in the supernatants (Fig. 3C, lanes 1 and 2, Western) indicating that the UBF protein had been quantitatively immunoprecipitated from the cell extracts by the anti-UBF antibody. The results from 4 separate experiments revealed that the increase (4.1 ± 0.7-fold) in radioactivity associated with contracting cardiomyocytes was not significantly different from the increased amounts (3.9 ± 0.6-fold) of UBF protein recovered from those cells. These results demonstrate that there was no net change in the specific radioactivity of UBF between contractile-arrested and contracting cardiomyocytes.

Transcriptional Regulation of UBF—The cellular content of UBF can be regulated by alterations in the rate of transcription from the UBF promoter (26). Accordingly, we performed North-
ern blots and nuclear run on assays to measure the steady-state levels and the rate of synthesis of UBF mRNA in arrested and contracting cardiomyocytes (Fig. 4).

Within 3 h of the onset of contraction, the amount of UBF mRNA began to rise (Fig. 4A, lane 3). After 12 h of contraction, the UBF mRNA levels were maximal and remained elevated for up to 3 days (Fig. 4B). This temporal pattern of UBF mRNA induction parallels that observed for the elevation in UBF protein in response to contraction. When similar blots were probed for mRNA encoding glyceraldehyde-3-phosphate dehydrogenase, no significant increases were observed (see Fig. 5A for an example). The kinetics of the increase in UBF mRNA levels indicated that at least part of this response might reflect increased expression of the UBF gene. Indeed, nuclear run-on assays demonstrated that nuclei isolated from cells that had been contracting for 12 h demonstrated a 4.5 ± 0.5-fold (n = 3) greater rate of UBF gene transcription than nuclei isolated from time-matched control cells (Fig. 4C). The specificity of the hybridization was demonstrated by the lack of hybridization of the de novo synthesized transcripts to control pUC19 DNA (Fig. 4C). Under the same conditions, transcription of the β-tubulin gene was unchanged, indicating that the transactivation of the UBF gene was not merely the result of a global increase in general transcription (Fig. 4C).

Stimulation of UBF Expression by Contraction Is Not Medi-
ated by Cardiac Non-cardiomyocytes or Angiotensin II—It was most likely that the contraction-induced increases in UBF were due to a direct effect on the cardiomyocytes themselves. However, it was also possible that regulation of UBF expression by contraction may require the permissive action of growth factors produced by the 5–10% contaminating population of cardiac non-cardiomyocytes which include fibroblasts and cells of the vasculature (38).

To distinguish between these possibilities, we measured the hypertrophic response of contracting cardiomyocytes purified by centrifugation through Percoll gradients (28). The results presented in Fig. 5 demonstrate that when cardiomyocytes purified in this manner were allowed to spontaneously contract, the induction of UBF mRNA (Fig. 5A, lane 3) was not significantly different from that observed in the standard cardiomyocyte preparations (Fig. 5A, lane 4). The results from 3–5 separate experiments were quantified and are presented in Fig. 5B. Thus, the alteration of UBF expression observed following contraction occurred in the hypertrophying cardiomyocyte population and required neither additional cell types nor the permissive action of paracrine growth factors from the cardiac non-cardiomyocytes. Moreover, under the same conditions, the mRNA levels of the “housekeeping” gene glyceraldehyde-3-phosphate dehydrogenase were not altered in response to contractile activity (Fig. 5A, lanes 1–4, lower panel), further demonstrating that the increase in UBF mRNA following initiation of contraction was not simply part of a nonspecific transcription response.

The physiological parameters of spontaneous contraction have not been defined, but they are likely to include both passive stretch and increased tension. It has been suggested that increased cardiomyocyte growth and some alterations in gene expression induced by passive stretch may be, at least partly, mediated by the autocrine release and action of angiotensin II (39). Accordingly, the possibility that angiotensin II might mediate the contraction-induced increases in cardiomyocyte growth and UBF expression in the present experiments was considered. The ability of contraction to augment protein accumulation after 48 h was the same in the presence and absence of the specific angiotensin II receptor antagonist, DuP 753 (1 μM), 137% and 135% of control, respectively. DuP 753 also failed to prevent contraction-induced increases in UBF mRNA (Fig. 5C) or protein (results not shown). Similar results were obtained with the alternative angiotensin II antagonist [Sar1,Val2]angiotensin II (1 μM). Both of these antagonists abolished short-term, angiotensin II-mediated increases in MAP kinase activity in contraction-arrested cardiomyocytes, indicating that they were active at the concentrations used (data not shown). Therefore, it is unlikely that the ability of contractile activity to modulate growth and UBF expression requires the permissive action of angiotensin II in our neonatal cardiomyocyte culture system.

The UBF Promoter Contains Elements Responsive to Contraction—Nuclear run-on assays demonstrated that the increased accumulation of UBF mRNA during contraction-induced hypertrophy was due to increased levels of transcription from the UBF gene. These results suggest that the UBF gene must contain cis-acting elements which respond to the signal transduction cascade initiated by contraction. To test this hypothesis, we transfected arrested and contracting cultured neonatal cardiomyocytes with various UBF/CAT constructs and compared the levels of CAT activity expressed (Fig. 6, Table I). As demonstrated in Fig. 6A, contracting cardiomyocytes transfected with a fragment of the UBF gene extending from −3124 to +93 (pCAT3.2) (+1 being the transcription initiation site) demonstrated a 3–4-fold increase in CAT activity as compared to noncontracting cardiomyocytes. On the other hand, a construct that extended from −665 to +93 (pCAT0.75) did not respond to contraction. These results suggest that the UBF gene contains a cis-acting element that is capable of responding to contraction and that it lies between −3124 and −665. Subsequent experiments with a more extensive set of 5′ deletion mutants (Fig. 6B) demonstrated that this element lies between −1189 and −665 of the UBF gene. In addition, these experiments suggest that the region between −2441 and −1189 is capable of repressing the induction of transcription from the UBF gene, as deleting this segment resulted in an amplification of the response to contraction from 3–4-fold to 16–18-fold.
Regulation of UBF in Cardiomyocytes by Contraction

Increased ribosome biogenesis is essential to the accumulation of protein during neonatal cardiomyocyte hypertrophy (9, 12, 34, 40). This process is regulated largely as the result of alterations in the transcriptional rate of the ribosomal RNA genes (rDNA) (12, 40). However, the molecular signals and pathways by which hypertrophic stimuli affect an increase in the rate of rDNA transcription in these cells are not known. For instance, it is possible that diverse hypertrophic stimuli augment rDNA transcription by activating common regulatory factor(s). Alternatively, they might act through divergent signaling pathways culminating in the activation of rDNA transcription through distinctly different sets of activators.

We have reported previously that the accelerated rDNA transcription associated with norepinephrine-induced cardiomyocyte hypertrophy was accompanied by increased cellular levels of the rDNA transcription factor UBF (13). We hypothesized that increased amounts and/or post-translational modification of UBF, i.e. increased UBF activity, might be a common mechanism by which diverse hypertrophic stimuli effect changes in rDNA transcription in cardiomyocytes. In order to address this question, an alternative model of cardiac hypertrophy was examined, one in which the cardiomyocytes were hypertrophying in response to spontaneous contraction (6–8). Specifically, we have compared the levels and enzymatic activity of RNA polymerase I and the content of the rDNA transcription factor, UBF, in contraction-arrested and contracting cultured neonatal cardiomyocytes. We have found that concomitant with contraction-induced increases in rDNA transcription, the cardiomyocyte level of UBF also increased in the absence of changes in the amounts or activity of RNA polymerase I. Immunoprecipitation of 32P-orthophosphorylated UBF demonstrated that the accumulated UBF was phosphorylated and thus transcriptionally active. Northern blots and nuclear run-on assays revealed that the accumulation of UBF was at least partially regulated at the level of transcription of the UBF gene. Further, transient transfection assays demonstrated that the UBF gene contained an element that could direct increased levels of transcription in response to contraction.

Regulation of UBF Content during Hypertrophy—The results presented here and those of our previous studies indicate that both spontaneous contraction and norepinephrine increase rDNA transcription in neonatal cardiomyocytes (11, 12). However, neither of these stimuli modulates the enzymatic activity or levels of RNA polymerase I in these cells (13). Thus, at least in response to these two fundamentally different hypertrophic stimuli, alterations in the amount or activity of RNA polymerase I cannot account for the observed changes in rDNA transcription. In direct contrast, both of these hypertrophic stimuli increased the cellular content of the rDNA transcription factor UBF (13). Moreover, the elevated level of UBF protein was proportional to the increased rates of rDNA transcription observed in response to each hypertrophic stimulus. For example, the maximal increases in UBF protein content and rate of rDNA transcription and total cellular protein content observed in contracting cardiomyocytes was 3.5–4.5-fold and 190–210%, respectively, compared to 2–3-fold and 150–175%, respectively, observed in response to norepinephrine (13).

UBF can stimulate rDNA transcription in vitro transcription reactions (10, 11, 18). In preliminary studies, we have found that the overexpression of UBF1 in primary neonatal cardiomyocyte cultures increases transcription from a co-transfected rDNA promoter in a dose-dependent manner. When considered together with these findings, our present data strongly implicate changes in the amount of UBF as a common mechanism by which diverse hypertrophic stimuli effect increases in rDNA transcription in neonatal cardiomyocytes.

Transcriptional Regulation of UBF by Contraction—Our observation that the rate of UBF gene transcription and the cellular contents of the UBF mRNA are increased relatively soon following initiation of contraction, and are maintained at these elevated levels, supports the hypothesis that UBF is contributing to the elevated rate of rDNA transcription. However, it is interesting to note that there are temporal differences in the way different hypertrophic stimuli regulate UBF mRNA content. For example, while the levels of UBF protein remained elevated during hypertrophy induced by adrenergic agonists (13), the elevation in cardiomyocyte UBF mRNA levels was transient. In contrast, spontaneous contraction is associated with a sustained elevation in the cardiomyocyte UBF mRNA levels. Thus, the differences in the transcriptional regulation of UBF by spontaneous contraction and norepinephrine may ultimately account for the quantitative differences in the ability of these two stimuli to increase UBF protein levels and, as a consequence, to trans-activate the rDNA promoter.

Contraction Is a Direct Stimulus for UBF Expression and Increased rDNA Transcription—It has been shown that a number of growth factors, such as basic fibroblast growth factor and transforming growth factor β or a transforming growth factor β-like growth factor, are released from the cardiac fibroblasts and that these factors can modulate cardiomyocyte growth and gene expression (41 and reviewed in Ref. 42). However, in our hands, cardiomyocyte cultures, purified by Percoll gradient centrifugation, exhibited the same response, i.e. the induction of UBF mRNA, as observed in cultures containing a minor

**Table I**

| pUBF/CAT construct | CAT | β-Galactosidase | | | | |
|--------------------|-----|----------------|---|---|---|---|
| Arrested 0.44 | 0.30 | 50.1 | 2.2 | 0.60 | 82.4 | |
| Contracting 0.44 | 0.27 | 38.6 | 0.2 | 0.71 | 97.8 | |
| Arrested 0.75 | 0.18 | 46.8 | 1.3 | 0.39 | 54.0 | |
| Contracting 0.75 | 0.19 | 35.2 | 1.1 | 0.53 | 74.0 | |
| Arrested 1.3 | 0.32 | 48.4 | 0.2 | 0.65 | 90.4 | |
| Contracting 1.3 | 0.55 | 40.1 | 0.3 | 13.8 | 1902.8 | |
| Arrested 2.5 | 0.32 | 44.8 | 1.4 | 0.72 | 99.3 | |
| Contracting 2.5 | 1.05 | 36.0 | 0.2 | 2.90 | 403.1 | |
| Arrested* 3.2 | 0.31 | 42.1 | 0.8 | 0.73 | 100 | |
| Contracting 3.2 | 0.90 | 32.4 | 0.9 | 2.80 | 382.0 | |

*a Set as the control value.

[4] R. D. Hannan, V. Y. Stefanovsky, T. Moss, and L. I. Rothblum, submitted for publication.
contaminating population of cardiac non-cardiomyocytes. Thus, the observed increase in UBF reflects a direct effect of contraction upon the cardiomyocytes and does not require the permissive release of growth factors from the cardiac non-cardiomyocytes. Moreover, our experiments with the angiotensin II antagonists DuP 753 and [Sar\(^1\),Ile\(^2\)]angiotensin II would obviate either an autocrine or paracrine role for angiotensin II in the induction of rDNA transcription and hypertrophy associated with contraction. In broader terms, this work is supportive of previous studies both in isolated perfused hearts and cultured cardiomyocytes (3, 6–8, and reviewed in Refs. 40 and 43) which have implicated a direct role for mechanical stimulation in the regulation of cardiac gene expression and protein synthesis.

Molecular Signals Regulating UBF Gene Expression—Second messengers and molecular signals that have been implicated in the coupling of hypertrophic growth stimuli with increased rDNA transcription and cardiomyocyte hypertrophy include classical and novel protein kinase C isoforms and the mitogen-activated protein kinase (MAPK) cascade. In an attempt to determine the relevant importance of such pathways to contraction-induced increases in UBF expression and cardiac hypertrophy, we conducted experiments with a number of appropriate kinase inhibitors including staurosporine, H7, and compound 3. However, while such kinase inhibitors did attenuate the contraction-mediated increase in UBF mRNA content and cardiomyocyte hypertrophy (results not shown), we found, as others have reported (14), that they also significantly inhibited the contractile activity of neonatal cardiomyocytes. Consequently, it was not possible to determine whether the altered regulation of UBF mRNA content was a direct effect of kinase inhibition or merely secondary to the decrease in contraction. Thus, it is unlikely that this approach will lead to the elaboration of the signal transduction pathways that couple contraction to increased UBF content and accelerated rDNA transcription.

An alternative approach to this problem would start with the analysis of the UBF promoter and the determination of the cis-acting elements and trans-acting factors responsible for increased levels of transcription in response to contraction. The UBF gene has been cloned, and the region immediately 5' to the transcription initiation site has been sequenced (37). In the studies described here, a UBF promoter/CAT chimeric gene transfected into neonatal cardiomyocytes responded to contraction in a quantitatively similar manner as the endogenous gene. Moreover, we have identified a region between –1189 and –665 relative to the predicted start of transcription of the UBF gene which contains an element(s) that mediates the response to contraction. Further studies using this approach will allow us to define the specific cis-acting elements within this region that regulate contractile-mediated UBF expression and to determine the respective trans-acting factors which bind to those sequences. These studies should put us one step closer to elucidating the signal transduction pathways that link the cascade initiated by contraction with accelerated rDNA transcription during neonatal cardiomyocyte hypertrophy.

Acknowledgments—We thank Drs. David Carey, Joseph Cheung, and Howard Morgan for their helpful comments on this manuscript.

\(^5\) R. D. Hannan, J. Luyken, and L. I. Rothblum, unpublished data.