Chromatin remodelling of the cardiac β -myosin heavy chain gene

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To investigate the role of chromatin structure in cardiac gene expression, we used the DNase I and micrococcal nuclease to probe the chromatin structure of the hamster cardiac β -MyHC gene. Two cardiac-specific DNase I hypersensitive sites (DHS) were identified, one of which was mapped to the -2.3 kb (β -2.3 kb) region and the other to the proximal promoter region of the β -MyHC gene. The two sites were readily detectable using nuclei from neonatal hamster heart; however, the proximal promoter site disappeared when adult hamster heart nuclei were used, and the -2.3 kb site decreased in intensity. We were able to demonstrate the gradual disappearance of this proximal

promoter DHS by comparing heart nuclei isolated from animals at late-gestation and 1-day-old stages. Furthermore, injecting thyroid hormone caused the disappearance of the proximal promoter DHS in late gestational fetal ventricular nuclei. Digestion of nuclei from various tissues by micrococcal nuclease revealed that the β -MyHC gene proximal promoter exists in an array of three specifically-positioned nucleosomes only in fetal heart chromatin. The β -MyHC gene proximal promoter is DNase I hypersensitive within one of the nucleosomal particles. Our data suggest that chromatin structure may participate actively in cardiac gene expression.

INTRODUCTION

The cardiac β -MyHC is one of two isoforms of MyHC expressed in vertebrate cardiac muscle. Its expression is tissue- and developmentally-specific, and it is regulated primarily at the transcriptional level [1,2]. In the cardiac ventricles of rodents, such as the mouse, the cardiac β -MyHC gene is expressed during embryonic and fetal development. At about the time of birth a switch in cardiac MyHC gene expression takes place, characterized by a down-regulation of the β -MyHC gene expression together with an up-regulation of the α -MyHC gene expression. In the adult, slow-twitch skeletal muscles continue to express the β -MyHC gene. Regulation of this gene is also influenced by pathological processes; for example, cardiac hypertrophy causes the re-expression of the β -MyHC gene in the ventricles of rodents [3].

Tissue- and developmentally-specific gene expression is regulated by the interplay between chromatin structure and transcription factors [4]. As recent studies have highlighted, transcriptional regulation can only be understood in the context of chromatin structure [5,6]. We have previously characterized and completely sequenced the hamster cardiac α - and β -MyHC genes [7,8]. Using this data we are now exploring the role of chromatin structure in the regulation of cardiac-specific gene expression. In this report, the chromatin structure of the cardiac β -MyHC gene was probed by two different nucleases, DNase I and micrococcal nuclease (MNase). We found that the proximal promoter region of this gene is DNase I hypersensitive within a nucleosomal particle defined by MNase digestion.

MATERIALS AND METHODS

Isolation of nuclei from tissues and DNase I digestion

Nuclei were isolated from freshly excised liver and heart ventricle of adult (3-month-old), 3-day-old, neonatal (1-day-old) and fetal

(late gestation) Syrian hamsters [10]. The digestion of nuclei with DNase I was performed as previously described [11]. Briefly, the nuclei were suspended in a buffer containing 15 mM Tris (pH 7.5), 60 mM NaCl, 0.5 mM spermidine and 0.15 mM spermine, treated with DNase I (Pharmacia) at concentrations ranging from 1 μ g to 10 μ g/ml at 37 °C for 2 min in the presence of 5 mM MgCl₂, subsequently disrupted in 1 M NaCl solution containing proteinase K (200 μ g/ml) and 0.25% SDS and incubated overnight at 37 °C. Genomic DNA was then extracted three times with phenol and chloroform and precipitated with sodium acetate and ethanol.

Micrococcal nuclease digestion of nuclei

Freshly prepared nuclei were resuspended in 95 μ l nuclear buffer (60 mM KCl, 15 mM NaCl, 5 mM MgCl₂, 0.1 mM EGTA, 15 mM Tris/HCl, pH 7.4, 0.5 mM DTT, 0.1 mM PMSF, 300 mM Sucrose). A serial dilution (4, 2, 1, 1/2, 1/4, 1/8 units/ μ l) of micrococcal nuclease was freshly made in 5 μ l nuclear buffer plus 0.4 mM CaCl₂. The nuclei were digested by micrococcal nuclease at 25 °C for 3 min, and the reactions were stopped by adding one volume of stop solution (20 mM EDTA, 1% SDS). The nuclei were then disrupted in 1 M NaCl plus proteinase K (200 μ g/ml), and digested overnight at 37 °C. The purification of genomic DNA was performed as described above.

Indirect-end labelling method [12]

The probe used in this study was amplified by PCR, using the cosmid DNA containing the 5' upstream region of the hamster β -MyHC gene as template. The amplified PCR fragment was subcloned into the TA vector (from Invitrogen) and verified by dideoxy DNA sequencing using an automated DNA sequencer (Pharmacia). The position of probe A adjacent to NcoI restriction

Abbreviations used: CAT, chloramphenicol acetyltransferase; CBP, CREB-binding protein; DHS, DNase I hypersensitive site; MNase, micrococcal nuclease; MyHC, myosin heavy chain; N-CoR, nuclear receptor co-repressor; TH, thyroid hormone; T₃, triiodothyronine; T₄, thyroxine; TR, thyroid hormone receptor; TRE, thyroid hormone response element.

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Figure 1 Structure of the hamster cardiac β -MyHC gene

The position of probe A is shown. DHSs are indicated by arrows. The positions of the two Nco I (N) restriction sites in β -MyHC gene are -5269 and +1722, respectively. The inset table summarizes the appearance of the DHSs identified in this study in various developmental stages of the β -MyHC gene.

Table 1 Serum level of free T_3 and T_4 in pregnant hamsters at late gestational stage

Serum T_3 and T_4 levels of timed pregnant hamsters were measured by a standard clinical radioimmunoassay, and expressed as mean $\pm$ SD. Student's *t* test indicated that both free T_4 and T_3 levels are significantly different between the control and the L-thyroxine-treated animals ($p < 0.01$).

	Serum free- T_3 (pmol/l)	Serum free- T_4 (pmol/l)
L-Thyroxine ($n = 4$)	11.5 ± 0.5	> 77.0
Control ($n = 4$)	4.5 ± 0.1	20.0 ± 1.0

site is indicated in Figure 1. The genomic DNA isolated from section one or two was then digested by NcoI and fractionated by 1 to 1.4% (w/v) agarose gels. The gel was then transferred to nitrocellulose and hybridized with the probe, which was prepared by random primer labelling. The specific activity of the probe was $0.5\text{--}1 \times 10^8/\mu\text{g}$. The nitrocellulose was then washed in $0.1 \times \text{SSC}$ and 0.1% SDS at 65°C for 20–45 min and exposed to film with an intensifying screen at -80°C for 2–7 days.

Animal model of hyperthyroidism [13]

Hyperthyroidism was induced in pregnant hamsters by administration of L-thyroxine (1 mg/kg/day, intramuscularly) between 7 and 13 days gestation (the gestation period of hamsters is 14 to 15 days). The animals were sacrificed on day 14 of the gestation period. Serum levels of free T_4 and T_3 were measured by a standard clinical radioimmunoassay (Table 1). The isolation of fetal heart ventricular nuclei and analysis of DHSs were as previously described.

Transfection experiments

Primary cardiomyocyte cultures were prepared from 1-day-old neonatal rat (Wistar) ventricular myocardium, as described previously [14]. The cells were grown for 36 h, then the CAT

construct (15 μg) containing the proximal β -MyHC gene promoter (-294 to +97) and pRSV β -galactosidase control plasmid (10 μg) were introduced into cells using the calcium phosphate precipitation method [15]. After 6 h, the cells were washed with $1 \times \text{PBS}$ and fresh media was added. After another 48 h, the cells were assayed for CAT and β -galactosidase activities [16,17]. For each CAT assay, equal amounts of protein were assayed (100 μg), and the reactions were allowed to proceed for 2 h. The CAT assay results were quantitated using the Bio-Rad phosphorimager, and the mean for the three transfections was plotted after adjusting for β -galactosidase activity. The CAT assays were repeated with at least three separate preparations of cells.

RESULTS

Tissue- and developmentally-specific DHSs of the cardiac β -MyHC gene

The position of probe A is shown in Figure 1. Using probe A, two strong cardiac-specific DHSs were detected within the 5 kb upstream regulatory region of the β -MyHC gene (Figure 2A, lane 5). These sites were not found in the genomic DNA extracted from neonatal liver nuclei (lanes 6 to 8) or in protein-free genomic DNA digested by DNase I (lanes 1 to 2). Based on the size of DNA fragments detected in the gel, and on the position of probe A, as shown in Figure 1, these two DHSs were mapped to -2.3 and -0.2 kb, respectively. The -0.2 kb DHS appeared to be located within the proximal promoter region of the β -MyHC gene. Using nuclei from various cultured cells, we also observed that these two DHSs only occur in cardiomyocytes [18].

The patterns of the DHSs in the β -MyHC gene during various developmental stages are shown in Figure 2(B). When nuclei isolated from adult hamster hearts were used, the strong DHS of the β -MyHC gene proximal promoter completely disappeared, and the -2.3 kb site became weaker (Figure 2B, lanes 1 to 3). The gradual disappearance of this proximal promoter DHS was demonstrated by comparing nuclei isolated from cardiac ventricle of late-gestation fetus (Figure 2C, lanes 1 to 3) and 1-day-old hamster (lanes 4 to 6).

It might be argued that the lower intensity of the proximal promoter DHS observed in the neonatal heart chromatin was caused by the overdigestion of nuclei by DNase I (Figure 2C, lane 4). However, we thought it unlikely that the neonatal heart chromatin in lane 4 is overdigested. While the intensity of both the major band and the -2.3 kb sub-band in the neonatal heart chromatin (lane 4) is comparable to the intensity of the fetal heart chromatin at the same concentration of DNase I (lane 1), a decrease of approximately 50% in the intensity of the proximal promoter DHS was observed in the neonatal heart chromatin (lane 4). The intensity ratios of the major band, the -2.3 kb sub-band and the proximal promoter sub-band in lane 4 (the neonatal nuclei) relative to lane 1 (the fetal nuclei) are 1.04, 0.9 and 0.54, respectively. This observation suggests that there exist intrinsic differences in DNase I sensitivity between the fetal and neonatal heart chromatin in the β -MyHC gene proximal promoter region.

The variation of DNase I concentrations used in the present study is owing to variations in purity of the cardiac nuclei preparations. Since cardiac nuclei are prone to be trapped in connective tissues during nuclei isolation, the yield rate of our nucleus preparation is frequently unsatisfactory. Throughout the course of the present study, therefore, the cardiac nuclei had to be prepared repeatedly. Fluctuations in purity between each nucleus preparation were inevitable. Although time-consuming to perform repeated cardiac nuclei isolations, we performed these studies and demonstrated the reproducibility of our findings.

Figure 2 DHSs of the cardiac β -MyHC gene

Nuclei from neonatal heart (panel **A**, 3–5; **B**, lanes 4–6), neonatal liver (**A**, lanes 6–8; **B**, lane 7), and adult heart (**B**, lanes 1–3) were treated with increasing concentrations of DNase I at 37 °C for 2 min as indicated at the top of the gel. Purified genomic DNAs (25 μ g) were digested with Nco I, electrophoresed on a 1% agarose gel, and subjected to Southern blot hybridization with probe A. Lanes 1 and 2 (naked DNA) of **A** are protein-free heart DNA digested with 0.05 and 0.01 μ g/ml DNase I, respectively, at 23 °C for 30 s. (**C**) The DHSs of the β -MyHC gene in the heart ventricle of late gestation fetal (FH, lanes 1 to 3) and neonatal stages (Neo, lanes 4 to 6) were compared. Restriction enzyme digestion of purified genomic DNAs (20 μ g) and Southern blot analysis with probe A were performed as described above. The lambda phage digested by Bst EII was used as a DNA marker in panels **A**, **B** and **C**.

Thyroid hormone causes disappearance of the proximal promoter DHS

Since the expression of the β -MyHC gene can be down-regulated by thyroid hormone [13,19,20], we asked whether the administration of thyroid hormone has any effect on the chromatin

Figure 3 Thyroid hormone alters the DHSs of the cardiac β -MyHC gene

DHS pattern of the β -MyHC gene in late gestational fetus treated with thyroid hormone (+T, lanes 3–5) were compared with an untreated group (–T, lanes 1–2). Each lane was loaded with 20 μ g genomic DNA purified from ventricular nuclei treated with DNase I as indicated. Subsequent Southern blot analysis with probe A was performed as above described. The lambda phage digested by Bst EII was used as a DNA marker in this study.

structure of the β -MyHC gene. As shown in Figure 3, injecting thyroid hormone into timed-pregnancy hamsters caused the disappearance of the β -MyHC gene proximal promoter DHS in the ventricular nuclei isolated from late gestational fetus. In this blot, no significant change in intensity of the β -2.3 kb site was observed, indicating that thyroid hormone has a major effect on the proximal promoter DHS. Consistent with our results, it has been indicated that the thyroid hormone response element (TRE) is adjacent to the TATA box of the β -MyHC gene [1]. However, the exact location of the TRE of the β -MyHC gene has not yet been defined.

The β -MyHC gene proximal promoter DHS is located on a specifically-positioned nucleosome defined by micrococcal nuclease digestion

To further investigate the chromatin structure of the cardiac β -MyHC gene, late-gestation fetal heart nuclei were digested by MNase. Electrophoretic analysis of nuclear MNase-digested samples revealed the expected nucleosome repeat length due to partial chromatin digestion (Figure 4A). These purified genomic DNAs were further analysed by the indirect-end labelling method with the same probe used in Figure 2. As shown in Figure 4(B), a prominent nucleosome repeat was observed in the late-gestation fetal liver (Figure 4B lanes 4 to 7) digested by MNase, but not in the naked DNA samples digested by MNase (lanes 1–3). According to the position of probe A (Figure 1) and DNA markers (lane 11) used in this study, this nucleosome repeat was mapped to the transcribed region of the β -MyHC gene.

However, in the late-gestation fetal heart (during which stage the gene is actively transcribed), the characteristic nucleosome repeat became less prominent in the transcribed region (Figure 4B lanes 8–10). Instead, three specifically-positioned nucleosomes (defined by MNase cleavage) around the proximal promoter region were detected only in the late-gestation fetal heart nuclei. It was found that the β -2.3 kb site in fetal heart nuclei is also sensitive to MNase digestion (Figure 4B lanes 8–10).

Interestingly and reproducibly, inclusion of a DNase-I-treated late-gestation fetal heart nuclear sample on the Southern blot showed that the β -MyHC gene proximal promoter region is

Figure 4 Tissue-specific nucleosome positioning over the β -MyHC promoter

Nuclei from late-gestational fetal liver and heart ventricle (FH) were treated with increasing concentrations of MNase where indicated. (A) Electrophoretic analysis of MNase-digested samples (5 μ g DNA) revealed the expected nucleosome repeat length owing to partial chromatin digestion (L: liver, lanes 1–4; FH: fetal heart, lanes 5–7). The DNA marker (Pharmacia) in lane 8 is a 100 bp DNA ladder. (The first 100 bp band is not shown in this figure because of its low intensity.) (B) Indirect end-labelling analysis of chromatin samples digested by MNase in A. Genomic DNA (20 μ g) was digested with NcoI, electrophoresed in a 1.4% agarose gel, transferred to nitrocellulose and hybridized to the probe A shown in D. Lanes 1 to 3 (naked DNA) are protein-free genomic DNA digested by 0.2, 0.4 and 0.8 U/ μ l of MNase for 5 min at room temperature, followed by indirect end-labelling analysis. Mr represents the specific DNA marker used in this study (B, lane 11; C, lane 5), which was generated by digestion of genomic DNA with NcoI + PstI (2013 bp; –294, PstI position) or NcoI + XbaI (1622 bp; +97, XbaI position). (C) Different batches of late-gestational fetal heart nuclei were digested by MNase (lanes 1–3), or DNase I (lane 4) and the purified genomic DNA (15 μ g) was analysed by indirect end-labelling methods as described in B. Lane 4 (D: DNase I) contains late-gestational fetal genomic DNA purified from nuclei treated with DNase I (3 μ g/ml). The proximal promoter DHS is indicated by a 'J' at the side of lane 4 (C). The positions of MNase cleavage in various samples are indicated by dots at the side of each lane. (D) Summary of MNase and DNase I cleavage sites over the β -MyHC promoter in late-gestational fetal heart chromatin. The position of nuclease cleavage are shown by arrows; inferred positions of nucleosomal particles are shown by the closed circles and given the names N1–N3. Because the N2 particle is DNase I hypersensitive and is likely to be a perturbed structure, its circle is shown as a dotted line. The *cis*-acting elements located within +1 to –294 are also shown (see text for references). M-CAT, 5'-CATTCCT-3'.

DNase I hypersensitive exactly between the micrococcal cleavages that define the N2 particle (Figure 4C lanes 1 to 4, also see Figure 4D). Thus, the N2 particle, if it is nucleosomal (as indicated in this study), may be perturbed. The position of each nucleosome particle relative to the transcription-initiation site is summarized in Figure 4(D). Since the resolution of MNase cleavage followed by indirect end-labelling analysis is relatively low, the exact position of each nucleosome in this proximal promoter region remains unclear. However, TATA box (–37 to –32) is estimated to be located within the N2 particle. The *cis*-acting elements

within (or near) the N2 particle are also shown in Figure 4(D). These elements have been identified previously by footprinting analysis (e.g. β e4 to β e6) [21], transient transfection assays (e.g. M-CAT DNA-binding sites) [22,23] and transgenic studies (e.g. Sp1) [24].

The proximal promoter DHS of the β -MyHC gene confers its tissue-specific expression

A transient transfection assay was performed to examine the

Figure 5 The proximal promoter DHS is able to confer tissue-specific expression of the chimaeric CAT construct

A representative CAT assay is shown. The CAT reaction mixture was extracted with ethyl acetate and analysed by thin-layer chromatography as described previously [15]. Myo, cardiomyocytes; Fib, fibroblasts. In lanes 1 and 3, cells were transfected with pSV40 CAT; in lanes 2 and 4, cells were transfected with β -294 CAT construct.

Table 2 Transient transfection analysis of the hamster cardiac β -MyHC gene promoter DHS site/CAT construct (β -294 CAT)

Myo, rat neonatal cardiomyocytes; Fib, cardiofibroblasts. The mean $\pm$ SD of three independent transfections is shown. The percent incorporation of acetylated form in each transfection had been normalized for β -galactosidase activity. Student's *t* test was used to assess the difference in CAT activity between the cardiomyocytes and cardiofibroblasts. The β -294 CAT activity in cardiomyocytes is significantly higher than the activity in the cardiofibroblasts ($p < 0.01$).

CAT Constructs	Myo	Fib
pSV40	64.2 $\pm$ 3.7	60.8 $\pm$ 4.8
β -294	19.2 $\pm$ 4.7	1.21 $\pm$ 0.3

function of the β -MyHC gene proximal promoter DHS. As a positive control, pSV40 CAT (chloramphenicol acetyltransferase) containing the SV40 promoter and enhancer (Promega) was transfected into cardiomyocytes or cardiofibroblasts, and the representative CAT assay is shown in Figure 5. As shown in Figure 5 and Table 2, while the positive control showed similar CAT activity in both cell types, significantly higher CAT activity was observed when the β -294/CAT construct (containing the proximal promoter DHS of the β -MyHC gene, see Figure 4C, lanes 4–5) was transfected into cardiomyocytes than when transfected into the cardiofibroblasts. This data suggests that the proximal promoter DHS is able to drive a high level of tissue-specific expression of the chimaeric CAT construct. Consistent with our data, Thompson et al. [21] demonstrated the tissue-specific expression of the cardiac β -MyHC gene in a transient transfection analysis, using a series deletion of rat cardiac β -MyHC gene promoter/CAT chimaeric constructs. In that study, the activity of the CAT reporter gene (–299 upstream of the transcription-initiation site) in cardiomyocytes is significantly higher than that of the HeLa cells and 3T3 cells.

DISCUSSION

In earlier studies, transcriptional regulation of the cardiac β -MyHC gene has been demonstrated by northern blot [25], CAT assay [21,22,26,27] and nuclear run-on analysis [28]. In the present study we extend those earlier investigations, and we

provide the first evidence that the down-regulation of the cardiac β -MyHC gene immediately after birth is accompanied by an alteration of the nucleosomal structure of its regulatory elements. Such changes in DHS patterns during cardiogenesis strongly suggest a role for chromatin remodelling in the regulation of cardiac-specific gene expression.

Thyroid hormone (TH) was found to have an effect on chromatin structure in the present study. Several lines of evidence indicate that its nuclear receptor, TR, may have a direct effect on chromatin structure. For example, Wong et al. [29] demonstrated that in the absence of thyroid hormone, TR-RXR (9-*cis* retinoic acid receptor) can bind to nucleosomal DNA, locally disrupt histone–DNA contacts and generate a DHS. Chromatin-bound unliganded TR-RXR silences transcription of the *Xenopus* TR β A gene within a canonical nucleosomal array [30]. When TH is added, the receptor directs further disruption of the chromatin structure over several hundred bases of DNA [30]. These results clearly indicate that nucleosomes have a role in both the repression and the activation of transcription by TR. Furthermore, evidence directly linking TR to chromatin remodelling comes from the discovery that its co-activators, p300/CBP, are histone acetyl-transferases [31,32], and its co-repressor, N-CoR (nuclear receptor co-repressor), is one of the components of a histone deacetylase complex [33–35].

In the present study, the chromatin structure of the cardiac β -MyHC gene was also probed by MNase. As shown in Figure 4, the usual nucleosomal repeat in the transcribed region becomes less prominent in fetal hearts, indicating that nucleosomal organization has changed. This result is consistent with the observation that the expected nucleosomal repeat of some heat shock genes is replaced by a continuous smear when intense transcription begins [9].

By contrast, the 5' proximal promoter region of the β -MyHC gene is organized in an array of specifically-positioned nucleosomes when it is actively transcribed. Interestingly, the promoter DHS site is mapped to one of the nucleosomes (N2 particle), implying that the N2 particle is perturbed. Many transcription-factor-binding sites crucial for the regulation of this gene (Figure 4D) happen to be localized in this nucleosome. Our results are consistent with a recent observation that a distal enhancer of the albumin gene exists in an array of precisely positioned nucleosomes only in liver chromatin [36]. Therefore, in future studies we propose to use the β -MyHC promoter organized into the nucleosome structure as a model to study the re-expression of β -MyHC gene in pathological conditions.

We would like to thank Dr. P. Y. Wong for his assays for serum T₃ and T₄ levels. This work was supported by the Ontario Heart and Stroke Foundation and the Medical Research Council of Canada. W.-Y. H. was supported by a University of Toronto Open Fellowship.

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