Myocardial Chromatin Activation in Experimental Hyperthyroidism in Rats

Role of Nuclear Non-Histone Proteins

Constantinos J. Limas and Christine Chan-Stier

SUMMARY Experimental hyperthyroidism induced in rats by daily injections of 20 μg of 3',3',5'-triiodothyronine (T3) resulted in a prompt increase in cardiac weight and RNA content. The mechanism of the RNA synthesis stimulation was studied by comparing template activity of myocardial chromatin from rats treated with T3 for 7 days with chromatin from euthyroid controls. Hyperthyroidism resulted in significant enhancement of chromatin template activity (184 ± 7.1 pmol 3H-UMP/mg per minute vs. 106 ± 4.8 pmol 3H-UMP/mg per minute; P < 0.001) and a significantly higher (102% more) number of transcription initiation sites. Dissociation of chromatin and subsequent reconstitution with nucleoproteins from both hyperand euthyroid rats demonstrated that the non-histone nuclear fraction (NHPs) accounted for the differences in RNA synthesis between the two groups. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoretic patterns of NHPs were similar for the two groups, but NHPs from hyperthyroid rats exhibited significantly higher degrees of phosphorylation because of higher nuclear protein kinase activity (71 ± 2.6 μmol P_i/mg per minute vs. 37 ± 1.9 μmol P_i/mg per minute in controls, P < 0.01). In vitro stimulation of RNA synthesis by NHPs was enhanced by the addition of protein kinase and cyclic adenosine 3',5' monophosphate findings suggest that stimulation of RNA synthesis in the myocardium of hyperthyroid rats is mediated by the nuclear NHPs and is dependent on phosphorylation of these proteins by nuclear protein kinases.

ADMINISTRATION of thyroid hormones leads to a moderate degree of cardiac hypertrophy which involves predominantly the myocardial cells, with little or no increase in interstitial tissue, possibly because of an inhibitory effect on collagen synthesis. Cardiac hypertrophy is a direct result of the stimulation of protein synthesis by the thyroid hormones; inhibition of protein degradation also may contribute to cardiac enlargement. This form of cardiac hypertrophy is of interest because contractility is preserved or even enhanced in contrast to other forms of cardiac hypertrophy that are associated with depression of myocardial performance.

The present report focuses on some aspects of cardiac RNA synthesis during the development of hypertrophy in response to triiodothyronine (T3). Increased transcrip- tive capacity of myocardial chromatin is an early event and appears to be related to changes in the regulatory properties of the non-histone chromosomal proteins.

Methods

Experiments were carried out on male Wistar rats weighing 200–250 g. T3 was injected daily (20 μg/100 g, ip) for 3–14 days; saline-injected rats served as controls.

Isolation of Nuclei

Cardiac tissue from pooled rat hearts was chilled and finely minced with scissors. All subsequent steps of the isolation were performed in the cold. The minced tissue was homogenized in five volumes of 0.32 M sucrose-3 mM MgCl₂ with a Polytron PT-20 homogenizer. The homogenate was filtered through a double gauze, and cold deionized water was added to the filtrate to reduce the total sucrose concentration to 0.25 M. Samples of the homogenate were taken for determinations of total DNA and protein content. The remainder was transferred to centrifuge tubes, and 0.25 ml of 0.32 M sucrose-3 mM MgCl₂ was pipetted into each tube to form a layer beneath the broken cell suspension. The tubes were centrifuged at 1,000 g for 10 minutes. The crude nuclear pellet was resuspended in 10 volumes of 2.4 M sucrose-1 mM MgCl₂, and the nuclei were sedimented by centrifugation at 100,000 g for 1 hour. Samples of the nuclear pellet were taken for determination of DNA and protein content.

Isolation of Chromatin

Chromatin was prepared by lysing nuclei in 80 volumes of triple glass-distilled water with several gentle strokes of a wide clearance Teflon homogenizer. The chromatin was allowed to swell in an ice bath for 20 minutes, pelleted by centrifugation at 12,000 g for 15 minutes, redispersed in distilled water by homogenization, and again pelleted by centrifugation at 12,000 g for 15 minutes.

Dissociation of chromatin was carried out according to the method of Levy et al. Nonsheared chromatin at concentrations of 0.6 mg DNA/ml was adjusted to contain 6 M urea, 0.3 M guanidine HCl, 0.1% β-mercaptoethanol, and 0.1 M sodium phosphate buffer (pH 7.0). DNA was then pelleted by centrifugation at 100,000 g at 4°C for 48 hours. More than 90% of the proteins of sheared chromatin were present in the supernatant extract following such sedimentation of DNA. The residual proteins remaining bound to DNA could be extracted further by extensive dialysis of the suspended pellet against 2%
SDS-0.1% α-mercaptoethanol-0.01 M sodium phosphate buffer (pH 7.0), followed by a similar sedimentation at 20°C. The supernatant extract was applied to a Bio-Rex 70 column equilibrated with 6 M urea-0.3 M guanidine HCl-0.1 M phosphate buffer (pH 7.0). The initial, nonabsorbed peak contains the non-histone proteins (Fig. 1). The column was washed with the equilibrating buffer until the absorbance at 230 nm of the eluate returned to baseline, and then the histones were eluted in one peak with a step of 4 M guanidine HCl in the equilibrating buffer.

DNA for reconstitution experiments was prepared by the Marmur technique supplemented with treatment with pancreatic ribonuclease A (50 μg/ml for 30 minutes at 37°C), and two extractions with phenol and chloroform-isooamyl alcohol. Chromatin reconstitution was carried out by the gradient dialysis technique as described by Huang and Bonner.7 DNA, histones, and non-histone proteins were combined in 3 M NaCl-5 M urea-0.01 M Tris (pH 8.3) to remove the urea, and then against 0.01 M Tris (pH 8.3) containing successively decreasing amounts of NaCl. After complete removal of sodium chloride, the reconstituted chromatin was pelleted by centrifugation at 20,000 g for 30 minutes, resuspended in 0.01 M Tris (pH 8.3), and repelleted by centrifugation at 20,000 g for 30 minutes.

**Polyacrylamide Gel Electrophoresis**

Chromatin samples (500 μg DNA as chromatin) were solubilized in 1 ml of 1% SDS-1% β-mercaptoethanol-0.01 M sodium phosphate (pH 7.0) at 22°C. Sucrose was added to a final concentration of 15%, and the samples were heated in boiling water for 5 minutes. Fifty-microliter samples containing 50 μg of chromosomal proteins were electrophoresed on 7.5% polyacrylamide gels containing 0.1% SDS for 7 hours at 8 mA/gel in a running buffer of 0.1% SDS-0.1 M sodium phosphate-5 mM EDTA (pH 7.0). The gels were fixed overnight at 37°C in 0.25% Coomassie blue in 40% ethanol-7% acetic acid, destained in 10% ethanol-7% acetic acid, and dried and counted in 10 ml of toluene-based scintillation fluid.

**Protein Kinase Assay**

Phosphorylation of non-histone proteins was carried out at 37°C in a reaction volume of 0.6 ml containing 10 mM Tris-Cl (pH 7.5), 15 mM magnesium acetate, 0.4 mM [γ-32P]ATP (New England Nuclear; sp act, 7.5 Ci/mmol), and 0.2 mg of non-histone proteins. Incubation was carried out for 10 minutes and was terminated by adding 3.0 ml of unlabeled 1 mM ATP, followed by 3.3 ml of cold 10% TCA-3% sodium pyrophosphate prior to filtration through nitrocellulose membrane filters (0.45 μm) presoaked in 1 mM ATP. Each filter was washed twice with 5 ml of cold 5% TCA-1.5% sodium pyrophosphate, oven-dried, and counted in 5 ml of toluene-based scintillation fluid.

Nucleic acids were extracted from the ventricles of T3-treated rats by a modification of the procedure described by Fleck and Munro.10 RNA was estimated from the absorbance at 260 nm, and DNA was measured by the diphenylamine reaction.11 Hydroxyproline was determined by the Woessner method,12 and protein, according to the method of Lowry et al.13

**Results**

T3 administration resulted in moderate cardiac hypertrophy as evidenced by an increased heart-body weight ratio (529 ± 4.0 vs. 400 ± 3.2 × 10^-6), left ventricular weight (132 ± 8.1 mg vs. 101 ± 9.3 mg), and RNA content (3.1 ± 0.05 mg/g vs. 2.0 ± 0.06 mg/g) in...
comparison to euthyroid controls. Hydroxyproline content, in contrast was essentially unchanged (2.52 ± 1.5 μg/mg dry weight vs. 2.40 ± 0.1 μg/mg in controls).

Chromatin isolated by the procedures described resulted in protein-DNA ratios of 1.94 for the T₃-treated and 1.72 for the euthyroid rats. This ratio was preserved in reconstituted chromatin (Table 1). In addition, the relative amounts of histones and non-histone proteins were similar in the two preparations: non-histone-histone ratio was 1.18 ± 0.09 in native, and 1.20 ± 0.10 in reconstituted, chromatin. Further evidence of the fidelity of chromatin reconstitution was obtained from the electrophoretic patterns of chromosomal proteins from native and reconstituted chromatins. A comparison of Figure 2, A and B, clearly establishes that, within the limits of resolution afforded by SDS-polyacrylamide gel electrophoretic fractionation, the relative amounts of protein in the principal molecular weight classes of chromosomal proteins (histones and non-histone proteins) associated with the genome of native and reconstituted myocardial cell chromatin are quantitatively and qualitatively indistinguishable. Figure 3 demonstrates that there are no major differences in electrophoretic patterns between non-histone proteins from hyperthyroid and euthyroid rats.

Native and reconstituted chromatin and DNA were transcribed under conditions in which the template is rate-limiting and reinitiation is prevented. Unsheared chromatin was used, since shearing enhances transcription in a nonspecific manner. Figure 4A shows the absence of significant variations in the transcriptional properties of native and reconstituted chromatin. These findings suggest that in both preparations a similar number of sites on the DNA is available for initiation and the time course of elongation is similar. Figure 4B demonstrates that native chromatin from hyperthyroid rats shows significantly higher transcriptive activity as well as an increased number of RNA chain initiation sites.

To examine the basis for these differences, chromatin from both experimental and control animals was dissociated and reconstituted by interchanging histone and non-histone protein mixtures. Table 2 demonstrates that reconstituted chromatin from control rats approximated transcriptive capacity of hyperthyroid rats only when NHPs from the latter groups were used; conversely, hyperthyroid chromatin lost a significant proportion of its transcriptive capacity when reconstituted in the presence of euthyroid NHPs.

Confirmation of the higher capacity of NHPs from hyperthyroid rats to stimulate myocardial RNA synthesis in vitro is given in Figure 5A. Previous studies by us¹⁴ and others ¹⁵ have suggested that the action of NHPs on gene transcription is related to their reversible phosphorylation by endogenous protein kinases. This possibility was explored in the two experimental groups. As shown in Figure 5B, significantly higher protein kinase activities were found in the hyperthyroid rats. Cytosol protein kinase activities on the other hand, were not different in

### Table 1

<table>
<thead>
<tr>
<th>Chromatin preparation</th>
<th>Protein-DNA (wt/wt)</th>
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<tbody>
<tr>
<td>Native euthyroid</td>
<td>1.72 ± 0.07</td>
</tr>
<tr>
<td>Reconstituted euthyroid</td>
<td>1.76 ± 0.06</td>
</tr>
<tr>
<td>Native hyperthyroid</td>
<td>1.99 ± 0.09</td>
</tr>
<tr>
<td>Reconstituted hyperthyroid</td>
<td>1.90 ± 0.01</td>
</tr>
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Histones were extracted with 0.25 N HCl. Nucleic acids were hydrolyzed with 1 N HClO₄ at 90° for 30 minutes, and the non-histone proteins were solubilized in 1 N NaOH. The amount of protein was assayed by the method of Lowry et al.¹³ and DNA, by the diphenylamine reaction. Results represent mean ± sem for six experiments.
FIGURE 4  A: Transcription of native (●) and reconstituted (O) myocardial chromatin carried out under conditions in which the template is rate-limiting, as described in the text. B: Transcription of native chromatin from hyperthyroid (●) and euthyroid (O) rats; each value represents mean ± SEM from six different preparations.

FIGURE 5  A: Stimulation of myocardial RNA synthesis by non-histone proteins from control (O) and hyperthyroid (●) rats. The assay medium contained 0.05 M Tris-HCl (pH 7.9), 0.01 M MgCl₂, 0.15 mM each of ATP, CTP, and GTP, 0.15 mM [γ-3H]-UTP, 50 µg DNA, and variable amounts of non-histone proteins in a final volume of 0.5 ml. The reaction was carried out for 10 minutes at 37°C and was terminated with 3 ml of 10% TCA. The precipitate was collected on 24-mm Whatman GF/C glass fiber filters and was washed four times with 3 ml of 2% TCA prior to drying and counting in 10 ml of toluene-based scintillation fluid. Results are given as mean ± SEM for seven separate preparations. B: Phosphorylation of myocardial non-histone proteins from euthyroid (●) and hyperthyroid (○) rats in the presence of [γ-32P]-ATP. Results are given as mean ± SEM for six preparations.

TABLE 2  Transcripitive Capacity of Myocardial Chromatin Reconstituted with Histones and Non-Histone Proteins of Varying Origin*

<table>
<thead>
<tr>
<th>Chromatin preparation</th>
<th>RNA synthesis (pmol [3H]-UMP/mg per minute)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control histones + control NHPs</td>
<td>106 ± 4.8</td>
</tr>
<tr>
<td>Control histones + hyperthyroid NHPs</td>
<td>159 ± 6.7</td>
</tr>
<tr>
<td>Hyperthyroid histones + hyperthyroid NHPs</td>
<td>184 ± 7.1</td>
</tr>
<tr>
<td>Hyperthyroid histones + control NHPs</td>
<td>111 ± 7.4</td>
</tr>
</tbody>
</table>

* As described in text.
Table 3: Effect of Protein Kinase-Induced Phosphorylation on Myocardial RNA Synthesis

<table>
<thead>
<tr>
<th>Condition</th>
<th>RNA synthesis (pmol H-UTP/mg per minute)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>118 ± 8.2</td>
</tr>
<tr>
<td>Control + PK</td>
<td>182 ± 7.3</td>
</tr>
<tr>
<td>Control + PK + cyclic AMP</td>
<td>201 ± 9.5</td>
</tr>
<tr>
<td>Hyperthyroid</td>
<td>196 ± 9.7</td>
</tr>
<tr>
<td>Hyperthyroid + PK</td>
<td>242 ± 8.4</td>
</tr>
<tr>
<td>Hyperthyroid + PK + cyclic AMP</td>
<td>298 ± 9.9</td>
</tr>
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</table>

Cyclic AMP-dependent protein kinase (100 μg) was added to the standard assay medium with or without 1 μM cyclic AMP. Results are given as mean ± SEM for seven experiments.

Figure 6: SDS-polyacrylamide gel electrophoresis of myocardial non-histone proteins from euthyroid (○) and hyperthyroid (●) rats phosphorylated in the presence of γ-32P[ATP].

The synthesis of RNA followed by stimulation of RNA polymerase and later by an increase in the rate of protein synthesis and oxygen consumption. On the basis of these observations, Tata proposed that the effects of thyroid hormone were caused largely by new protein synthesis brought about by enhanced transcription of DNA. This conclusion was supported by the finding that inhibitors of protein synthesis, such as actinomycin D and cycloheximide, could block the effect of the hormone.15

Binding of thyroid hormones to specific nuclear receptors has been reported by several workers.16-22 Occupancy of these receptors has been correlated with physiological responses to the hormones.19 Although the chemical nature of these receptors is not entirely settled, most investigators assign them to the acidic non-histone nuclear proteins.19-22 In contrast to steroid hormones for which cytosol-nuclear traffic is necessary for action, entry of cytosol receptors does not seem to be necessary for thyroid hormones.23 Similarly, there is no change in the total number of nuclear binding sites.

We have examined the mechanism of genetic transcription in the hearts of hyperthyroid rats. Administration of T3 over 2-3 weeks leads to the development of moderate cardiac hypertrophy which involves cardiac myocytes almost exclusively. This may be due to a direct inhibitory effect of thyroid hormones on collagen biosynthesis3 and contrasts with the prominent increase in interstitial tissue seen with other forms of cardiac hypertrophy. It provides the advantage of justifiably ascribing changes in biochemical parameters to modification of cardiac cells alone. Preliminary experiments in our laboratory have shown changes in the regulation of chromatin transcription identical to those presented in this study, when isolated myocytes (instead of whole hearts) were used for the preparation of nuclei (unpublished observation).

The duration of T3-induced cardiomegaly was associated with enhanced chromatin template activity. Not only was the rate of RNA synthesis enhanced, but the number of initiation sites on the DNA template was increased, as shown by experiments in which only one cycle of RNA chain initiation was possible. These results indicated an augmentation to the transcription process by T3. We attempted to obtain more information about the chromatin component(s) responsible for the increased template activity. Such activity could be regulated either by a change in the template (DNA) or the associated nucleoproteins: the basic histones which act as repressors of genetic transcription or the acidic nonhistone proteins which have been implicated in the positive control of transcription in a variety of biological systems.26-27 To distinguish between these possibilities, chromatin from cardiac nuclei of control and T3-treated rats was dissociated and then reconstituted by using nucleoproteins and DNA from different sources. The results strongly suggest that the increased transcrip- tive capacity of chromatin from hyperthyroid rats is determined by the non-histone protein fraction number and are in agreement with recent studies indicating the important role of non-histone proteins in RNA synthesis regulation.27-29 These proteins have a number of properties that make them attractive candidates for the positive control of gene transcription.30 Thus, they are heteroge-

Table 4: Effect of Duration of T3 Administration on Myocardial RNA Synthesis and Protein Kinase Activity

<table>
<thead>
<tr>
<th>Days</th>
<th>RNA synthesis (pmol H-UTP/mg per minute)</th>
<th>Protein kinase (μmol Pi/mg per minute)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>102 ± 4.3</td>
<td>37 ± 1.9</td>
</tr>
<tr>
<td>3</td>
<td>181 ± 6.4</td>
<td>71 ± 2.6</td>
</tr>
<tr>
<td>7</td>
<td>183 ± 6.2</td>
<td>70 ± 2.2</td>
</tr>
<tr>
<td>14</td>
<td>153 ± 5.9</td>
<td>62 ± 3.1</td>
</tr>
<tr>
<td>21</td>
<td>148 ± 6.1</td>
<td>60 ± 2.7</td>
</tr>
</tbody>
</table>

RNA synthesis was assayed using myocardial chromatin (20 μg DNA), as described in the text. Protein kinase activity was determined at 37°C for 10 minutes, in the presence of 200 μg non-histone proteins.
neous, tissue-specific, bind to DNA, and stimulate RNA polymerase activity. We have recently confirmed these properties specifically for myocardial non-histone proteins. Their stimulatory effect on RNA synthesis seems to depend on two properties: (1) their ability to bind to homologous chromatin DNA and (2) to undergo reversible phosphorylation. The latter is related to the presence, in the myocardial non-histone nuclear fraction, of several cyclic AMP-dependent protein kinases. We have isolated nine discrete protein kinases by phosphocellulose chromatography of purified non-histone proteins and have shown both substrate specificity and differences in cyclic AMP-dependency. The ability of non-histone proteins to stimulate RNA synthesis was related directly to the degree of their phosphorylation by nuclear protein kinases. The existence in rat myocardial nuclei of multiple, cyclic AMP-dependent protein kinases has also been reported by Akhtar and Itzhaki. In view of the presence in nuclei of a non-histone protein phosphatase, a system is available for the control of gene transcription by reversible phosphorylation of non-histone proteins.

Neither the yield nor the electrophoretic pattern of myocardial non-histone proteins were changed by thyroxine administration. Thus, an increased concentration of nuclear acceptor sites for thyroxine probably was not present, in agreement with previous results obtained by using the rat liver. It still is possible, however, that an increased cytoplasmic-nuclear traffic on non-histone proteins constitutes an acute response to thyroxine administration that was missed because of the earliest observation was made 3 days following initiation of T4 administration.

The validity of the conclusions about the importance of non-histone nuclear proteins in regulating myocardial chromatin transcription in hyperthyroidism obviously depends, in large part, on the preservation of transcription fidelity during dissociation and reconstitution of chromatin. This issue has been examined recently, and it was shown that recovery of the subunit structure of chromatin after dissociation and reconstitution is markedly affected by the procedure used. When dialysis from high concentrations of salt and urea initially removes the urea and after dissociation and reconstitution is markedly affected pends, in large part, on the preservation of transcription fidelity during dissociation and reconstitution.

References

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