TAFII250, Egr-1, and D-type cyclin expression in mice and neonatal rat cardiomyocytes treated with doxorubicin

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1Lady Davis Institute for Medical Research and 2Department of Pathology, Sir Mortimer B. Davis Jewish General Hospital, Montreal H3T 1E2; and 3Division of Experimental Medicine, Department of Medicine, McGill University, Montreal, Quebec, Canada H3A 1A3

Saadane, Nacéra, Lesley Alpert, and Lorraine E. Chalifour. TAFII250, Egr-1, and D-type cyclin expression in mice and neonatal rat cardiomyocytes treated with doxorubicin. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H803–H814, 1999.—Differential display identified that gene fragment HA220 homologous to the transcriptional activator factor II 250 (TAFII250) gene, or CCG1, was increased in hypertrophied rodent heart. To determine whether TAFII250 gene expression is modified after cardiac damage, we measured TAFII250 expression in vivo in mouse hearts after injection of the cardiotoxic agent doxorubicin (DXR) and in vitro in DXR-treated isolated rat neonatal cardiomyocytes. In vivo atrial natriuretic factor (ANF), β-myosin heavy chain (β-MHC), Egr-1, and TAFII250 expression increased with dose and time after a single DXR injection, but only ANF and β-MHC expression were increased after multiple injections. After DXR treatment of neonatal cardiomyocytes, we found decreased ANF, α-MHC, Egr-1, and TAFII250 expression. Expression of the TAFII250-regulated genes, the D-type cyclins, was increased after a single injection in adult mice and was decreased in DXR-treated cardiomyocytes. Thus expression of Erg-1, TAFII250, and the D-type cyclins is modulated after cardiotoxic damage in adult and neonatal heart.

We hypothesized that an increase in TAFII250 expression might be present after cardiotoxic damage. In this paper the mouse sequences of the TAFII250 are compared with the relevant human and hamster sequences. We describe our experiments on the expression of TAFII250 and Egr-1 after DXR treatments given either acutely or chronically to mice or to isolated rat cardiomyocytes. We describe an association between the increase in TAFII250 expression and the increase in expression of the TAFII250-regulated genes, cyclins D1, D2, and D3, in vivo. In contrast, DXR-treatment of isolated neonatal rat cardiomyocytes decreased both cardiac damage; differential gene expression; transcriptional activator factor II 250; early growth response-1; cyclins D1, D2, and D3.

PREVIOUSLY, WE ISOLATED transgenic mice that expressed the polyomavirus large T-antigen gene in cardiomyocytes, testes, and endothelial cells (1, 6–8). The transgenic mice display a cardiomyocyte hypertrophy with morphological and molecular features found in rodent hypertrophy (17, 18). We set out to identify specific mRNAs overexpressed in the hypertrophied transgenic heart and low or absent in the normal heart. We compared the expression of a series of known genes in normal and hypertrophied hearts and used differential display to identify genes discordantly expressed in normal vs. hypertrophied rodent hearts. We identified early growth response-1 (Egr-1) as uniquely increased in expression in every hypertrophied heart and c-fos as increased in most hypertrophied transgenic hearts (17). Differential display (30, 35) identified a set of 10 DNA fragments representing mRNAs overexpressed in hypertrophied hearts from transgenic mice and low or absent in normal hearts. We identified one of the DNA fragments as homologous to the hamster and human transcription activator factor II 250 (TAFII250).

TAFII250, the largest protein in the TFIID complex, binds to the TATA-box binding protein (TBP) and participates in the formation of the TFIID complex (42). The TAFII250 protein has NH2-terminal and COOH-terminal serine kinase domains, a histone acetyltransferase (HAT) domain, two retinoblastoma (Rb) binding domains, an adenovirus early region 1A (E1A) binding domain, a nuclear localization signal, two bromodomains, and a non-histone chromosomal protein (HMG-1)-like area (50). TAFII250 was originally named cell cycle gene 1 (CCG1) because it was mutated in the temperature-sensitive (ts) G1 mutant hamster cell lines tsBN462 and ts13 (16). These tsBN462 and ts13 cells do not show a global loss of mRNA levels, but levels of cyclin A, cyclin D1, and cyclin D3 are reduced at the nonpermissive temperature (47, 53, 58). Because cell death could be suppressed by transfection of wild-type CCG1 into the ts cell lines, it was suggested that wild-type CCG1/TAFII250 functioned as a repressor of apoptosis (46, 58), linking TAFII250 expression to the control of cyclin expression and apoptosis.

Programmed cell death, apoptosis, occurs in the myocyte after ischemia-reperfusion, myocardial infarction, longstanding heart failure, normal cardiac development, and aging (20, 22). Cardiotoxic agents include the alkaloid emetine in ipecac syrup, cocaine, ethyl alcohol, and doxorubicin (DXR) (11). The anthracycline antibiotic is a widely used antineoplastic agent (reviewed in Ref. 48). However, the irreversible cardiotoxicity of DXR limits its therapeutic use. DXR-damaged hearts typically display a dilated cardiomyopathy characterized by a reduced ejection fraction, ventricular wall thinning, and chamber dilation. DXR-induced cardiac damage is thought to be due to induction of oxidative stress and apoptosis (48). In DXR-treated hearts, damaged cardiomyocytes are replaced by connective tissue, and the remaining cardiomyocytes experience hypertrophy.

We hypothesized that an increase in TAFII250 expression might be present after cardiotoxic damage. In this paper the mouse sequences of the TAFII250 are compared with the relevant human and hamster sequences. We describe our experiments on the expression of TAFII250 and Egr-1 after DXR treatments given either acutely or chronically to mice or to isolated rat cardiomyocytes. We describe an association between the increase in TAFII250 expression and the increase in expression of the TAFII250-regulated genes, cyclins D1, D2, and D3, in vivo. In contrast, DXR-treatment of isolated neonatal rat cardiomyocytes decreased both...
MATERIALS AND METHODS

Materials. Taq DNA polymerase and Superscript II reverse transcriptase polymerase were obtained from GIBCO BRL, a PTC-100 programmable temperature controller was purchased from MJ Research, and RNase H was purchased from MBI Fermentas. Klenow DNA polymerase, oligo(dT), and RNase H was purchased from PTC-100 programmable temperature controller was purchased from MJ Research, and RNase H was purchased from MBI Fermentas.

Screen Plus membrane was obtained from DuPont-New England Nuclear. CD-1 virgin and pregnant mice and Sprague-Dawley rat day 1 neonates were purchased from Charles River Canada. All oligonucleotides for differential display and gene-specific analyses were purchased from the Sheldon Biotechnology Centre at McGill University. Random primers, pd(NTP)6, were purchased from Pharmacia. Doxorubicin was a gift from Dr. Moulay Alaoui-Jamali, Lady Davis Institute for Medical Research.

Primers and cDNAs. The sequences of the anchored primers were 5'-T11CT, 5'-T11AC, 5'-T11AG, and 5'-T11AA. The sequences of three arbitrary 10-mer primers used in this study were 5'-CAGGATAGGC-3' (AP-1), 5'-CTGTAATGTCG-3' (AP-2), or 5'-CGTAAAGGT-3' (AP-3). Unless otherwise noted all primers were selected using the PC/GENE program (IntelliGenetics).

Mouse TAFII250-specific primers for the NH2-terminal kinase region were regions P4 (5'-AGGAAACAGCAGACGGAGACTAAGG-3') and M15 (5'-CCACAGCCGAGCAGTCCATAAGG-3') with an annealing temperature of 59°C, P5 (5'-GGGCTTCACAGGCATCCT-3') and M15 (5'-CGTACACTTCTACCTCTG-3') with an annealing temperature of 57°C. The cyclin D1 primers were cyclin D (5'-CTGCCAAGCAGGTGCACC-3'), cyclin D1 specific primers for the NH2-terminal kinase region were regions P4 (5'-AGGAAACAGCAGACGGAGACTAAGG-3') and M15 (5'-CCACAGCCGAGCAGTCCATAAGG-3') with an annealing temperature of 59°C, P5 (5'-GGGCTTCACAGGCATCCT-3') and M15 (5'-CGTACACTTCTACCTCTG-3') with an annealing temperature of 57°C. The cyclin D1 primers were cyclin D (5'-CTGCCAAGCAGGTGCACC-3'), cyclin D1 specific primers for the NH2-terminal kinase region were regions P4 (5'-AGGAAACAGCAGACGGAGACTAAGG-3') and M15 (5'-CCACAGCCGAGCAGTCCATAAGG-3') with an annealing temperature of 59°C, P5 (5'-GGGCTTCACAGGCATCCT-3') and M15 (5'-CGTACACTTCTACCTCTG-3') with an annealing temperature of 57°C. The cyclin D1 primers were cyclin D (5'-CTGCCAAGCAGGTGCACC-3'), cyclin D1 specific primers for the NH2-terminal kinase region were regions P4 (5'-AGGAAACAGCAGACGGAGACTAAGG-3') and M15 (5'-CCACAGCCGAGCAGTCCATAAGG-3') with an annealing temperature of 59°C, P5 (5'-GGGCTTCACAGGCATCCT-3') and M15 (5'-CGTACACTTCTACCTCTG-3') with an annealing temperature of 57°C. The cyclin D1 primers were cyclin D (5'-CTGCCAAGCAGGTGCACC-3'), cyclin D1 specific primers for the NH2-terminal kinase region were regions P4 (5'-AGGAAACAGCAGACGGAGACTAAGG-3') and M15 (5'-CCACAGCCGAGCAGTCCATAAGG-3') with an annealing temperature of 59°C, P5 (5'-GGGCTTCACAGGCATCCT-3') and M15 (5'-CGTACACTTCTACCTCTG-3') with an annealing temperature of 57°C.

In the acute study, 6- to 8-wk-old female CD-1 mice (average wt 25 g) were randomly placed into eight groups (n ≥ 8 mice/group) and injected intraperitoneally with DXR at concentrations of 10, 20, or 30 mg/kg, and the mice were killed 1 or 7 days later. Control animals were injected with saline.

In the chronic study 6- to 8-wk-old CD-1 female mice (average wt 25 g) were randomly placed into two groups (n ≥ 8 mice/group) and injected intraperitoneally three times at 1-wk intervals with either 10 mg/kg DXR or saline, and mice were killed 1 wk after the last injection.

Representative midventricle horizontal cross sections of all mouse hearts were fixed in neutral buffered Formalin, routinely processed, and embedded in paraffin. Sections were cut at 4 µm and stained with hematoxylin and eosin or with Masson's trichrome stain. Sections were examined in a blinded fashion.

Neonatal rat cardiomyocyte and cardiac fibroblast isolation and DXR treatment. Day 1 rat neonates were killed by decapitation, and the hearts were removed. Hearts were minced in pancreatin-containing media (GIBCO 25720–012), digested at 37°C, and briefly centrifuged, and the supernatant was discarded. Fresh pancreatin solution was added and the digestion was repeated five to six times until most of the heart tissue was digested. Once in growth medium [DMEM/199 (4:1), 5% fetal calf serum, 5% horse serum and antibiotics] the cell suspension was plated on petri dishes for 1.5–2 h to allow fibroblasts to adhere. The cardiomyocyte-enriched and fibroblast-depleted suspension was then plated on gelatin-coated dishes at a density of 1 x 10^5 cells/ml. Cardiac fibroblasts did not constitute more than 10% of the cardiomyocyte cultures. Beating cardiomyocytes were found the next day. Before DXR treatment, the media were removed and replaced with treatment media with [DMEM/199 (4:1), 1% horse serum and antibiotics] for 24 h. Plates were randomly assigned to saline or treatment regimens, and the media were removed and replaced with treatment media plus saline or treatment media plus 2 µM DXR. After 24 h, washed cells were scraped from the dishes and RNA was extracted.

RNA preparation. RNA from normal nontransgenic adult heart and hypertrophied transgenic hearts, as well as from DXR-treated and saline-treated hearts, was isolated after homogenization in guanidine isothiocyanate and centrifugation through a cesium chloride cushion (2). RNA was prepared from neonatal rat cardiomyocytes and fibroblasts by the method of Chomczynski and Sacchi (10). All purified RNAs were incubated with RNAase-free DNase (Pharmacia) to remove residual genomic DNA.

Differential display. The differential display technique was performed as described in Mou et al. (35). Briefly, 0.2 µg of DNA-free total RNA from nontransgenic and hypertrophied transgenic heart was incubated separately with 50 µM of each anchored primer (T11AC, T11AG, T11GT, and T11AA), 200 µM dNTPs, 10 mM DTT, and first-strand reaction buffer and Superscript reverse transcriptase II according to the manufacturer's (GIBCO BRL) instructions. Each PCR reaction con-
Results

Identification of overexpressed genes associated with heart hypertrophy. Differential display was used to identify hypertrophy-associated genes (Fig. 1). Different combinations of anchored primers and arbitrary primers were added to RNA prepared from adult hearts from nontransgenic mice and hypertrophied hearts from adult metallothionein-1 promoter-polyomavirus large T antigen (MT-PVLT) transgenic mice. Hypertrophied hearts from the MT-PVLT transgenic mice averaged 2.3 times heavier than hearts from nontransgenic hearts (7, 17, 18). Figure 1A shows results from a differential display using T11AG and AP-1, AP-2, or AP-3, respectively. Amplified DNA fragments present in some samples, but not in others, were identified by inspection; the corresponding piece of gel and Whatman paper were identified, cut out, and rehydrated in 150 µl of distilled water, and the DNA was eluted by boiling. The DNA was precipitated in ethanol and redissolved in water before reamplification. Denatured PCR-amplified fragments (~100 ng) were fixed to duplicate nylon membranes using a slot-blot manifold, and then the membranes were hybridized with double-stranded DNA (dsDNA) probes prepared from mRNA of another set of normal nontransgenic or hypertrophic transgenic hearts (6). Autoradiography was carried out at −80°C for up to 3 days. Positive differentially expressed fragments were cloned into either pCRII (Invitrogen) or a T-tailed EcoRV linearized Escherichia coli.

Both strands from inserts of three independent clones from the differential display or TAFII250 gene fragments were sequenced using a T7 DNA polymerase sequencing kit (Pharmacia). The gene fragment sequences were analyzed for similarity to those deposited in GenBank/EMBL DNA databases. Translation of mouse DNA sequences into amino acid sequences was performed with Gene Runner (Hastings Software).

RT-PCR. DNA-free total RNA (3 µg) was denatured with 250 ng of random primers at 70°C for 10 min. The first-strand reaction proceeded according to the Superscript II reverse transcriptase instructions and included RNase H digestion. The PCR contained Taq DNA polymerase buffer, 1 mM dNTP, MgCl2, gene-specific primers (0.6 µg), Taq DNA polymerase (2.5 U), and 2 µl of the first-strand reaction in 100 µl final reaction volume. The amount of MgCl2 was optimized for each primer pair. The amplification parameters were 94°C for 4 min, followed by 24 cycles of 94°C for 1 min, annealing for 1.5 min, and elongation at 72°C for 2–4 min, followed by a postamplification of annealing for 2 min and elongation at 72°C for 7 min. Control PCR reactions with all primer sets showed a linear response up to 30 cycles (data not shown). The annealing temperature for each primer pair is described in Primers and cDNAs. Aliquots of each PCR reaction were electrophoresed through 1.5% agarose, visualized by ethidium bromide staining, photographed, and transferred to Gene Screen Plus membrane using the alkaline downward transfer method (2). The reaction products were hybridized to radiolabeled gene-specific cDNAs, washed, and exposed to X-ray film for 2–10 min. Each series of RT-PCR reactions was repeated at least three separate times. Suitably exposed X-ray films were scanned using an HP Scanjet 5100C and HP Precision Scan software (Hewlett-Packard). The areas under the peaks were quantitated using Scion Image Release Beta 3 software (National Institutes of Health, Bethesda, MD). Means ± SE and P values were calculated using StatViewSE + and ANOVA, respectively.

Mouse TAFII250. TAFII250 is a multidomain protein. The relative locations of the NH2-terminal and COOH-terminal kinase domains, separate Rb and adenovirus E1A binding domains, histone acetyltransferase domain, and RAP74 binding domain are shown in Fig. 2A (50). The mouse nucleotide HA220 sequence (GenBank accession no. AF081115) matched the hamster TAFII250 (hTAFII250) DNA sequence between 2,465 and 2,576 bp and the human TAFII250 (huTAFII250) DNA sequence between 2,507 and 2,618 bp, respectively, with 80–100% homology (49, 50). This area was identified as Rb binding area 2 (50). When the deduced amino acid sequence was evaluated (Fig. 2B) and mouse, hamster, and human sequences were compared, we identified a single mismatch, threonine to glutamine at position 849 of the hamster sequence.
The nucleotide sequences of huTAFII250, respectively, with nucleotide changes located sporadically throughout the sequence. When the amino acid sequence was deduced (Fig. 2C), we identified two clusters of differences among the mouse, hamster, and human sequences centered around haTAFII250 amino acids 275 and 330. An 872-bp region spanning the RAP74 and COOH-terminal kinase regions was amplified from mouse first-strand cDNA, extracted from an agarose gel, cloned into pBluescript, sequenced, and named mTAFII250-RC. The mTAFII250 nucleotide sequence (GenBank accession no. AF081117) was 92.5% and 89% homologous with the haTAFII250 and huTAFII250 sequences, respectively. A comparison among the mouse, hamster, and human amino acid sequences (Fig. 2D) identified four differences, an alanine-threonine sequence change from the proline-alanine sequence at positions 1,183 and 1,184 present in both hamster and human TAFII250 and a glutamine-methionine sequence change from the histidine-leucine sequence present in the hamster and human TAFII250 at positions 1,442 and 1,443. The conservation of the nucleotide and amino acid sequences in these three disparate regions of the gene suggests that the mouse, hamster, and human TAFII250 gene is highly conserved and further suggests that the mTAFII250 likely functions in an identical way to its better characterized human and hamster homologs.

mTAFII250 in DXR-treated mice: acute study. We first examined the time course of ANF and Egr-1 expression after a single injection of DXR. Animals (n = 4 per time period) were injected with DXR or saline and then killed on day 1, 3, 5 or 7. RT-PCR analyses of heart RNA showed that after a 10 mg/kg dose of DXR, female CD-1 mice reproducibly displayed an increase in ANF and Egr-1 expression that was detectable in some mice as early as day 3 and was present in all mice on days 5 and 7 after injection (data not shown). Tubulin expression was similar at all time points, indicating a specific rather than a global effect of DXR on cardiac gene expression. DXR-injured cardiomyocytes generally show myofibril loss with vacuolar formation and thin filament degeneration both in vivo and in vitro (3). Histological examination of hematoxylin and eosin- or trichrome-stained cross sections of DXR-treated midventricle heart did not show evidence of fibrosis, although vacuolar changes (arrows) were noted on day 7 that were consistent with DXR-induced cardiac damage (Fig. 3).

We examined the effect of different doses of DXR on TAFII250, Egr-1, and cyclin D, as well as the expression of the muscle-specific ANF, α-MHC, and β-MHC genes. Mice were treated with a single injection of DXR at doses of 10, 20, or 30 mg/kg and were killed 24 h or 7 days after injection. Mice treated with the 10 mg/kg...
The dose had no discernible change in behavior but showed less than one-half of the weight gain seen in the saline-injected control mice at 7 days. Mice treated with the 20 and 30 mg/kg dose averaged a weight loss approaching 6% at 7 days, were lethargic, and had a disheveled appearance. Heart-to-body weight ratios were calculated (mean 4.52, range 4.39-4.69) and did not differ significantly between control and treatment groups.

The effect of increasing doses of DXR on the expression of ANF, α-MHC, and β-MHC 24 h or 7 days after injection is shown in Fig. 4. The 10 mg/kg dose of DXR did not result in a rise in ventricular ANF initially at 24 h, whereas after 7 days, an increase in ANF mRNA is seen. Doses of 20 and 30 mg/kg induced a significant increase in ANF at both 24 h and 7 days after injection. β-MHC expression was not increased 24 h after injection of the 10 mg/kg dose of DXR and was significantly increased 7 days postinjection. At the higher doses of DXR an increase in β-MHC was also detected both 24 h and 7 days after injection. In contrast, expression of α-MHC was unaffected by the DXR injection. The expression of tubulin was used to standardize the level of expression and did not vary between saline- and DXR-injected mice. These results demonstrate a dose- and time-dependent response of the ventricular heart to DXR treatment. Furthermore, the results indicate that the expression of some genes, e.g., ANF and β-MHC genes, increases with time and dose of DXR, whereas the expression of other genes, e.g., tubulin and α-MHC genes, is unaffected.

We examined the expression of Egr-1, an immediate-early gene whose expression increases during hypertrophy, and TAFII250. The results are shown in Fig. 4. Egr-1 expression was similar to control samples after the 10, 20, or 30 mg/kg dose 24 h after DXR injection.
Seven days postinjection the expression of Egr-1 was elevated significantly at all doses tested. TAFII250 expression was not significantly (P > 0.05) increased 24 h after injection of the 10 mg/kg dose of DXR, whereas after the increased dose of 20 or 30 mg/kg, TAFII250 expression was similar to the saline-injected control. Seven days postinjection TAFII250 expression was significantly increased at all doses of DXR tested. A similar pattern of TAFII250 expression was found when PCR primers homologous to the region separating the RAP74 and COOH-terminal kinase were used to amplify mTAFII250 mRNA in the PCR reaction (data not shown). These results indicate that Egr-1 and TAFII250 were not increased shortly after DXR-injection but were elevated after 7 days.

mTAFII250 in DXR-treated mice: chronic study. Cardiotoxic damage is more likely if multiple doses and an increased accumulated dose of DXR are received (31, 52). Therefore we injected female mice three times with 10 mg/kg DXR spaced at 1-wk intervals, for an accumulated dose of 30 mg/kg. Mice were killed 7 days after the last injection. Control animals received an equal volume of saline. Saline-injected mice had an average weight gain of 1.6 g (range 0.7–2.3 g) over the injection period, whereas DXR-injected animals had an average weight loss of 2.7 g (range 1.5–3.9 g), suggesting that this schedule of chronic DXR treatment was detrimental to the overall health of the mice. Heart-to-body weight ratios did not differ between control (mean 4.47, range 3.95–4.98) and DXR-treated animals (mean 4.4, range 4.08–5.06), a result that was similar to the results noted already in the acute study. Histological analysis using hematoxylin and eosin staining as well as trichrome staining of midventricular cross sections indicates that, although vacuolar changes were found in cardiomyocytes, no evidence of fibrosis was visible.

We first examined RNA samples prepared from ventricular heart for tubulin, ANF, α-MHC, and β-MHC expression, with the results shown in Fig. 5. Tubulin expression was similar in the saline-injected and DXR-injected animals, indicating that basal gene expression was unaltered by the treatment. ANF expression was significantly increased in the DXR-treated animals compared with the saline-injected animal. α-MHC expression was similar in the saline-injected and DXR-treated animals. In contrast, β-MHC expression was increased in the DXR-treated compared with the saline-injected control. These results indicate that, similar to our findings in the acute study, chronic treatment with DXR caused a selective increase in ANF and β-MHC expression, whereas expression of tubulin and α-MHC was unaffected.

We next examined Egr-1 and TAFII250 expression. Neither Egr-1 nor TAFII250 expression was signifi-
and DXR-treated cardiomyocytes or cardiac fibroblasts. ANF
Tubulin did not vary between the saline-treated or
b
Cardiomyocytes and cardiac
lated dose, 30 mg/kg.
initiating amount of DXR, 10 mg/kg, or the accumu-
lation of transcription. Expression from saline-injected mice was designated as
A RNA sources: Sal, saline-injected mice; DXR, mice injected with 10 mg/kg DXR. *Significantly different (P < 0.05)
from DXR-treated mice. Values are means ± SE.

Fig. 5. DXR chronic study. Southern blots of RT-PCR reactions were performed as described in Fig. 4 and in
MATERIALS AND METHODS using RNA prepared from ventricles of mice injected with either saline or 10 mg/kg DXR for
3 consecutive weeks with mice killed 1 wk after last injection; 6 animals/group were analyzed in triplicate. A: bar
graphs of Southern blot data from 3 separate experiments. Expression from saline-injected mice was designated as
1. RNA sources: Sal, saline-injected mice; DXR, mice injected with 10 mg/kg DXR. *Significantly different
from saline-injected mice. B: representative Southern blots from RT-PCR of RNA isolated from saline-injected and DXR-treated mice.

mTAFII250 in isolated neonatal rat cardiomyocytes and cardiac fibroblasts. Cardiomyocytes and cardiac
fibroblasts were isolated from day 1 rat neonates. Preparing of the pancreatin-treated heart cell suspension
greatly reduced the number of fibroblasts in the cardiomyocyte culture and allowed separate treatment of
both cardiomyocyte and cardiac fibroblasts cultures with saline or DXR. Inspection of the cultures showed
the presence of beating cardiomyocytes in the cardiac fibroblast culture and also a minor number of cardiac
fibroblasts present in the cardiomyocyte culture. Plates were randomly assigned to either saline treatment or
the addition of 2 µM DXR for 24 h before RNA collection. Cells were treated with media containing 1%
horse serum for 24 h before the addition of saline or DXR to minimize any increase in cell numbers for the
cardiac fibroblast relative to the cardiomyocyte cultures.

We examined the expression of tubulin, ANF, α-MHC, and β-MHC, and the results are shown in Fig. 6. Tubulin did not vary between the saline-treated or DXR-treated cardiomyocytes or cardiac fibroblasts. ANF and α-MHC, but not β-MHC, were significantly decreased in the DXR-treated cardiomyocytes compared with the saline-treated cardiomyocytes. These results suggest that DXR treatment of the neonate cardiomyocytes caused a decrease in expression of ANF and α-MHC without a significant decrease in tubulin or β-MHC expression.

We then examined the expression of Egr-1 and TAFII250 in the DXR-treated cardiomyocytes (Fig. 6).
Egr-1 and TAFII250 expression was significantly reduced in the DXR-treated cardiomyocytes and cardiac
fibroblasts compared with the saline-treated cardiomyocytes and cardiac fibroblasts.
TAFII250-regulated genes: the D-type cyclins.
TAFII250 was shown to positively regulate the expres-
sion of the D-type cyclins, D1, D2, and D3 (46, 49).
D-type cyclin RNA expression coincides with the level
of cyclin protein in cardiomyocytes (43). We therefore
examined the expression of cyclins D1, D2, and D3 by
using a competitive PCR method (55) in the acute
study, in the chronic study, and in isolated rat neonatal
cardiomyocytes and cardiac fibroblasts; the results are
shown in Fig. 7, A and B. Control experiments (data not
shown) analyzing the cyclin D-specific genes individu-
ally gave identical results to those shown here. One day
after DXR injection in the acute study (Fig. 7A), the
expression of cyclins D1, D2, and D3 was significantly
increased after the 10 mg/kg dose compared with the
saline-injected control. The 20 and 30 mg/kg doses
resulted in an increase in cyclin D1 and D2, but not
cyclin D3, expression. Seven days postinjection the
expression of cyclins D1, D2, and D3 was significantly
increased at the 10 mg/kg dose, and cyclins D1 and D2
were significantly increased at the 10 and 20 mg/kg
doses tested. We found that the 10 mg/kg dose elicited
larger increases in all D-type cyclin expression than the
20 or 30 mg/kg dose of DXR. In the chronic study only
an increase in cyclin D2 expression was found in the
saline-injected compared with the DXR-injected samples. Isolated cardiomyocytes (Fig. 7B) treated
with saline or DXR showed no change in cyclin D1
expression but significant decreases in expression of D2
and D3 cyclins. In contrast, DXR or saline treatment of
cardiac fibroblasts did not change the pattern or expres-
sion of D cyclins.

DISCUSSION
Synthesis of mRNA by RNA polymerase II is con-
trolled by DNA elements in the region upstream of the
coding sequence. Cis-acting DNA elements can gener-
ally be divided into basal promoter and enhancer regions (reviewed in Refs. 23, 28, 40, 57). Recent evidence suggests that the basal and enhancer regions communicate through activators that form part of the TFIID complex. TFIID includes the TATA-box binding protein and a set of eight or more transcription-associated factors (TAFs). These TAFs are not required for basal transcription but mediate regulated transcription (reviewed in Ref. 28). A current model suggests that TAFs form contacts with each other as well as with gene-specific activators and repressors (57). It has been hypothesized that TAFs play an active role in regulating cell proliferation, development, differentiation and apoptosis and may be involved in oncogenesis and genetic diseases (38). It is likely that properties present in TAFII250 help expose promoter elements in chromatin, permitting entry of the transcriptional machinery; 2) aid in the recruitment of RNA polymerase II; and 3) attract and hold enhancer binding proteins through protein-protein binding with other TAFs and transcription factors and intrinsic enzyme activities. Our results suggest that TAFII250 is an important regulator of gene expression in heart.

We have shown that TAFII250 and Egr-1 expression is increased in DXR-induced cardiac injury and that such overexpression is dose- and time dependent. We further show that the increase in TAFII250 is concomitant with an increase in expression of the D-type cyclins, previously shown to be TAFII250. To our knowledge this is the first time TAFII250 expression has been linked to a physiological process. In contrast, in neonatal cardiomyocytes, TAFII250 and D-type cyclin expression was decreased. These conflicting results suggest that expression of TAFII250 may be linked to that of the D-type cyclins in cardiomyocytes and also suggest that adult and neonatal cardiomyocytes do not respond to DXR similarly.

Circumstantial evidence has suggested a role for a protein with the activities present in TAFII250 in cardiac gene expression. A hallmark feature of cardiac hypertrophy and damage is the reexpression of previously silent muscle-specific genes (reviewed in Ref. 56). Acetylation of histones H3 and H4 has been correlated with chromatin transcriptional activation and release of specific promoters from repression (reviewed in Ref. 37). Recently, an H3- and H4-specific histone acetyltransferase activity was detected in vitro in the amino terminal half of TAFII250 (34). Studies in HeLa cells showed that an activating transcription factor/CAMP-response element (ATF/CRE) site and a novel DXR-
induced kinase were necessary and sufficient for DXR-mediated gene expression changes (27). ATF/CRE sites (TGACGTCA) are present in many gene promoters, such as ANF and Egr-1, that are induced in cardiac damage and hypertrophy (41, 54). TAFII250-dependent activation of cyclin D1 is lost when the CRE site is absent, suggesting that TAFII250 interacts, directly or indirectly, with CRE binding proteins (53).
treated HeLa cells, incubation with the H7 kinase inhibitor abolished DXR toxicity, suggesting that kinase activity is central to its toxicity (27). Kurabayashi et al. (27) concluded that DXR activates a kinase that potentiates the activity of a factor already bound to DNA. In the current model of TAF activation TAFII250 activates transcription by binding to TATA-box binding proteins previously bound to DNA (9, 12). The TBP-bound TAFII250 subsequently interacts with different TAFs, suggesting that TAFII250 and the transcription factors, including factors such as Sp1, interact directly (9, 12). TAFII250 has both an NH²-terminal and COOH-terminal serine kinase (42). Although phosphorylation was shown to be specific for RAP74 compared with other TAFs, TAFII250 phosphorylation of other, potentially adjacent and transcriptionally relevant targets, such as Egr-1, c-jun, or c-fos, has not been reported. It is unclear if the results with HeLa cells can be directly transcribed to cardiomyocytes, but the limited data using cardiomyocytes support the idea that regulation via ATF/CRE sites and kinase activation is important in DXR-mediated cardiomyocyte damage.

TAFII250 controls expression of the D-type cyclins in fibroblasts (47, 53, 58). We found that higher amounts of TAFII250 expression were associated with higher amounts of the D-type cyclins in adult hearts and lower amounts of TAFII250 expression were associated with lower amounts of D-type cyclins neonatal cardiomyocytes in vitro. These studies suggest that TAFII250 controls the level of D-type cyclin expression in heart. The role of cyclins and cdk activity in heart is unclear. In transgenic mice overexpressing cyclin D1 in the heart, an increase in cardiomyocyte DNA content occurred without an increase in cardiomyocyte number (51). No change in muscle-specific contractile proteins or Rb phosphorylation was detected, and the transgenic mice did not develop a hypertrophy or cardiomyopathy. Similarly to the transgenic experiments, we found no temporal correlation between ANF and β-MHC expression and TAFII250 or cyclin expression in chronically treated DXR-damaged hearts. These data do not preclude control of ANF or β-MHC expression by TAFII250 at some time point but indicate that a continued high level of TAFII250 is not necessary.

Cyclin D may play a role, other than in cell cycle regulation, in terminally differentiated cells. We found expression of all three D-type cyclins in neonatal rat cardiomyocytes, however, and found a significant decrease in cyclin D2 and D3, but not in cyclin D1, with DXR treatment. Also decreased were TAFII250, Egr-1, α-MHC, and ANF expression in the DXR-treated cardiomyocytes, whereas there was no change in tubulin or β-MHC levels. A decrease in expression of the cyclins in our experiments suggests that TAFII250 may regulate cyclin D expression in neonatal rat cardiomyocytes. However, similar results were not detectable in DXR-treated cardiac fibroblasts, suggesting that TAFII250 may not regulate cyclin expression in these fibroblasts. The results with the neonatal rat cardiomyocytes are similar to those found in the ts hamster fibroblast cell lines, in which expression of TAFII250 and cyclin A and D were linked (46, 58). In other studies with neonatal cardiomyocytes, serum and angiotensin II increased cyclin D1 and D3 expression without an increase in D1-associated enzyme activity (43). Studies with myoblast and myeloblastic differentiation indicate nonparallel expression of D1 and D3 cyclins, disassociation of cyclin amounts with cdk activity, and cyclin D association with non-cdk proteins (25, 26, 39). In our experiments we found the largest increase in expression of cyclin D1 in the DXR-treated animals at all doses in the acute study, whereas there was no change in cyclin D1 and a drop in cyclins D2 and D3 in the DXR-treated in vitro cardiomyocyte cultures. These data suggest that the D-type cyclins have nonredundant functions and that the role of cyclin D proteins in undifferentiated cells may not be equivalent to that of differentiated cells and may change with development.

Egr-1 is a nuclear phosphoprotein with three zinc fingers that bind to the GC-rich element CGCCCGCGC, present in, e.g., α-MHC gene and the Egr-1 gene itself. Egr-1 modulates transcription through repressive and activating domains and in competition with Sp1 (reviewed in Ref. 13). v-Sis-dependent transformed cell growth in vitro and in vivo was suppressed by Egr-1 expression. Egr-1 is increased after ionizing radiation injury and oxidative stress, and cells transfected with a dominant-negative Egr-1 mutants show a decreased survival (15, 19, 20). In the heart, Egr-1 is increased transiently after endothelin, adrenergic, and ANG II activation and stretch, and also in our transgenic model of hypertrophy (Ref. 4, 32, 36, and 59; and unpublished data). Taken together these data suggest that Egr-1 is induced when cells are under stress and that the induction may play a protective role.

The present data demonstrate an increase in TAFII250 and Egr-1 expression on day 7 rather than immediately after an acute DXR injection on day 1. These data suggest that the TAFII250 and Egr-1 increase is a delayed response in the whole animal. Histological data indicate that vacuolar changes were seen in cardiomyocytes without fibrosis, demonstrating that major heart damage did not yet occurred during our experiments. Because of the demonstrated protective effect of Egr-1 in cultured fibroblast cells and the antiapoptotic activity of TAFII250 in ts hamster cells, their acute increase may serve to protect hearts from damage. The return to basal levels in the chronically treated hearts may indicate exhaustion of responsive cells, and thus a limit to any protective response, or that expression is transient and unrelated to a protective function. Preliminary data from ongoing experiments using DXR-treatment of Egr-1 knockout mice suggest that a lack of Egr-1 is associated with a worse physiological response to DXR treatment.

The response to an acute dose of DXR is not equivalent to the same dose given chronically or to the accumulated dose. Chronic dosage of DXR is not associated with increased DXR metabolism, and an increase in glutathione-S-transferase activity does not confer DXR resistance in mammary carcinoma cells (Ref. 45; Aloumi-J amali, unpublished data). DXR-induced free
radical formation is thought to be important in its cardiotoxicity, and transgenic mice overexpressing moderate increases in catalase activity in heart were more resistant to DXR toxicity (11, 24). The antioxidant enzymes glutathione peroxidase and glutathione reductase were decreased up to 12% in cardiac tissue after chronic DXR treatment without any change in superoxide dismutase or catalase activity (14). These data suggest that the chronically treated heart would be more sensitive to further oxidative challenges, and indeed we detected molecular changes, higher ANF and β-MHC levels, suggesting that damage had occurred. Despite the histological and molecular changes, the levels of Egr-1, TAFII250, and D1 and D3 cyclins were unchanged, and only a modest, but significant, increase in D2 cyclin was found in the DXR-injected animals in our chronic study. Further studies are required to resolve the mechanism of the different responses to acute and chronic exposure to DXR.

In conclusion, we show that Egr-1 and TAFII250 are increased in DXR-mediated damage and that TAFII250 overexpression is associated with an increased expression of the D-type cyclins in adult heart; TAFII250 underexpression is found along with decreased expression of cyclins in DXR-treated rat neonatal cardiomyocytes. Egr-1 expression follows a similar expression pattern to that of TAFII250. TAFII250 and the D-cyclin expression react similarly in DXR-treated adult heart and in DXR-treated neonatal cardiomyocytes, suggesting that TAFII250 regulates D-type cyclin expression in heart. The differences in direction of the gene expression changes in DXR-treated adult vs. neonatal cardiomyocytes suggest that developmental changes may alter the response of the heart to damage. Given 1) the bipartite kinase activity of TAFII250, 2) its activation of transcription via activating transcription factors, 3) its histone acetyltransferase activity (HAT), releasing chromatin repression of promoters, 4) its ability to form protein-protein complexes with other TAFs and enhancer-binding proteins, and 5) its overexpression in DXR-damaged heart shown here, we propose that TAFII250 is an important regulator of normal and pathological heart gene expression.

We thank Drs. John Th'ng and Moulay Alaoui-Jamali for critical reading of the manuscript. We thank Dr. Hung The Hyunh for use of Hewlett-Packard equipment and software in the densitometry. This work was supported by grants from the Medical Research Council of Canada (MT-13111) and the Heart and Stroke Foundation of Quebec (L. Chalifour). Address for reprint requests: L. E. Chalifour, Div. of Experimental Medicine, Dept. of Medicine, McGill University, 3755 Côte Ste. Catherine, Montréal, Québec, Canada H3T 1E2. Received 27 May 1998; accepted in final form 1 October 1998.

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