Characterization of Nucleophosmin (B23) as a Myc Target by Scanning Chromatin Immunoprecipitation*

Received for publication, September 5, 2001, and in revised form, October 5, 2001
Published, JBC Papers in Press, October 16, 2001, DOI 10.1074/jbc.M108506200

Karen I. Zeller‡, Timothy J. Haggerty§§, John F. Barrett§, Qingbin Guo, Diane R. Wonsey§§, and Chi V. Dang‡§§**

From the ¶Program in Human Genetics and Molecular Biology and §Department of Medicine, ¶The Johns Hopkins Oncology Center and ¶The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

The genetic program through which a specific transcription factor regulates a biological response is fundamental to our understanding how instructions in the genome are implemented. The emergence of DNA microarray technology for gene expression analysis has generated vast numbers of target genes resulting from specific transcription factor activity. We use the oncogenic transcription factor e-Myc as proof-of-principle that human genome sequence analysis and scanning of a specific gene by chromatin immunoprecipitation can be coupled to identify target transcription factor binding sequences. We focused on nucleophosmin, also known as B23, which was identified as a candidate Myc-responsive gene from a subtractive hybridization screen, and we found that sequences in intron 1, and not 5' sequences in the proximal promoter, are bound by e-Myc in vivo. Hence, a scanning chromatin immunoprecipitation (SChIP) strategy is useful in analyzing functional transcription factor-binding sites.

The e-myc gene, encoding a basic helix-loop-helix transcription factor, was first described more than 20 years ago as the cellular homolog to the v-myc oncogene. Several mechanisms including gene amplification, point mutations, and chromosomal rearrangements activate e-myc in human tumors. Myc mediates tumorigenesis through activation of genes involved in cell metabolism, proliferation, and apoptosis as well as repression of genes that may promote cellular differentiation and cell cycle arrest. Along with its heterodimeric partner Max, Myc binds to the canonical enhancer box (E box), 5'-CACGTG-3', as well as noncanonical sequences in the regulatory regions of target genes, activating transcription (1). Detailed mechanisms by which Myc represses transcription are yet unknown (1). Through subtractive hybridization (2), serial analysis of gene expression (3) and, most recently, cDNA microarray analysis (4, 5), the full spectrum of genes affected by e-myc is beginning to emerge. Although elucidation of the functions of these genes may prove insightful for defining the role of Myc in both normal and pathologic cell growth and proliferation, mechanisms by which Myc alters gene expression levels remain largely unstudied.

Numerous experiments strongly implicate a role for e-myc in promotion of cell growth (increase in cell size) that is independent of proliferation. A decrease in Drosophila Myc (d-myc) function results in small cell and body size, termed Minutes. In contrast, d-myc overexpression in the fly wing increases growth rate and cell mass (6). In all stages of the cell cycle, increased lymphocyte cell size and protein synthesis rates are observed in transgenic mice overexpressing e-myc in B cells (7). The role of myc in cell growth is further substantiated by observations that aberrantly high e-myc expression increases hepatocyte size in vivo and correlates with increased mRNA levels of genes involved in protein synthesis including translation initiation factors, aminoacyl-tRNA synthetases, ribosomal proteins, and nucleolar proteins involved in ribosomal biogenesis such as nucleolin (C23) (8–11). The link between the oncogenic effect of Myc and increased ribosomal biosynthesis is intriguing, in particular regarding the longstanding observation that a morphologic feature of neoplastic transformation is nuclear hypertrophy.

B23 is an abundant nucleolar phosphoprotein that interacts with C23 (12) and functions in the assembly and transport of ribosomes. Nucleic acid binding, ribonuclease, and molecular chaperone activities have been mapped on the B23 protein (13). Interestingly, B23 has also been shown to associate with unduplicated centrosomes. Upon phosphorylation by CDK2/cyclin E in late G1 of the cell cycle, B23 dissociates from centrosomes followed by initiation of centrosome duplication (14). Dramatic increases in B23 mRNA and protein levels are observed in tumorigenic cells and in cells stimulated with various mitogens (15, 16). Taken together, these studies reveal an important role for B23 in cell growth and proliferation.

Previous studies have shown that the expression of e-myc correlates with B23 expression (4, 5, 8). Wild-type Rat1 fibroblasts maintain a B23 transcript level 3.5-fold higher than fibroblasts bearing a homozygous deletion of myc (4). In myc-overexpressing avian bursal neoplasia, B23 transcript levels were 3.5-fold higher than in normal bursa (5). Adenoviral transduction of e-myc into mice led to dramatic increase in liver B23 mRNA, which correlated with increasing Myc protein levels 3–5 days post-infection (8). All of these studies indicate that increased myc expression results in elevated B23 transcript level but evidence for the direct regulation of B23 by e-myc is lacking.

Determining whether Myc directly or indirectly, through the induction of other transcription factors, regulates target genes is important in delineating the direct oncogenic effects of the Myc transcription factor (1). One approach taken to determine whether a Myc-responsive gene is a direct target is the use of the MycER system. However, these experiments do not reveal which cis-acting elements are important for transcriptional regulation by Myc. With the availability of the human genome
sequence and antibodies against specific transcription factors, one can identify important regulatory sequences by chromatin immunoprecipitation (ChIP) assays. With ChIP, formaldehyde-fixed chromatin is sheared and immunoprecipitated with a Myc antibody for example, and the resultant DNA assayed for specific DNA sequences by PCR.

Recently, ChIP has been employed to demonstrate the recruitment of c-myc or N-myc to promoters of known targets (17–19). However, these studies had not used ChIP to distinguish between potential binding sites in a candidate target gene. The human B23 locus, which contains a number of potential Myc-binding sites including two adjacent canonical (5'-CACGTG-3') sites in intron 1, is an ideal candidate for scanning with ChIP to identify Myc-associated DNA fragments. By using a quantitative ChIP assay to scan B23 and electrophoretic mobility shift assays, we determined that sequences in or around intron 1 are bound preferentially by Myc in vivo and in vitro. Luciferase reporter assays further reveal that the intron 1 sequence, in contrast to sequences upstream of the transcriptional start site, respond to Myc in transient transfections. Thus, our study identifies B23 as a direct c-myc target and provides proof-of-principle that the technique of scanning ChIP (SChIP), used with the emerging human genome data base, is effective in identifying transcription factor-binding sequences that are important for gene regulation.

EXPERIMENTAL PROCEDURES

Cell Culture—Rat1 fibroblasts expressing a fusion protein of Myc and the human estrogen receptor (MycER, a gift from J. M. Bishop, University of California, San Francisco) were grown in DMEM supplemented with 10% FBS (Life Technologies, Inc.), penicillin (200 units/ml) and streptomycin (200 µg/ml). For MycER induction, cells were grown to confluency and then serum-starved (DMEM, 0.1% FBS) for 72 h. A-4-Hydroxytamoxifen (4-OHTM) was added to a final concentration of 0.25 µM. To block protein synthesis, cycloheximide (CHX) was added to a final concentration of 10 µM 30 min prior to stimulation with 4-OHTM. TGR (wild-type rat fibroblast), HO105 (c-myc null fibroblasts), and HO105-myc (HO105 cells stably expressing a c-myc transgene) were maintained in DMEM supplemented with 10% FBS and antibiotics. For the serum stimulation experiments, confluent cultures were exposed to 0.25% serum for 3 h. To obtain cells expressed with wild-type c-myc, DMEM containing 10% FBS. Human ATCC 2091 (skin fibroblasts) cells were maintained in DMEM plus 10% FBS. For serum starvation, cells at 70% confluence were switched to DMEM containing 0.1% FBS and incubated for 48 h prior to re-stimulation with serum.

Total RNA Isolation and Northern Blot Analysis—Total RNA was isolated using Trizol (Life Technologies, Inc.). 10 µg of RNA was run on a 1.2% agarose gel, 2 h 143). The total products fell in the linear range of the total input DNA. Relative abundance of each B23 fragment was compared after normalization of the quantity of each fragment in the 2-h ChIP sample to the quantity in the total input DNA sample.

Reporter Assays—A 350-bp region from the human B23 gene (−274 to +b7, +1 is the transcriptional start site) that contains three putative c-myc binding sites was PCR-amplified and cloned into the pGL2-promoter vector (Promega). Forty-bp double-stranded oligonucleotides containing sequence from intron 1 of B23 with either wild-type (5’-CACGTG-3’) or mutant (5’-CCCCGGG-3’) boxes were synthesized (Life Technologies, Inc.) and cloned into the pGL2-promoter vector. To assess the ability of Myc to transactivate these luciferase reporter constructs, subconfluent NIH 3T3 fibroblasts were transfected with the Corel (gift of Nair and Burley) contain DNA binding domains for the activation domain of Myc. The human estrogen receptor (MycER, a gift from J. M. Bishop, University of California, San Francisco) were grown in DMEM supplemented with 10% FBS and antibiotics. For the MycER induction, cells were grown to confluency and then serum-starved (DMEM, 0.1% FBS) for 72 h. 4-Hydroxytamoxifen (4-OHTM) was added to a final concentration of 0.25 µM. To block protein synthesis, cycloheximide (CHX) was added to a final concentration of 10 µM 30 min prior to stimulation with 4-OHTM. TGR (wild-type rat fibroblast), HO105 (c-myc null fibroblasts), and HO105-myc (HO105 cells stably expressing a c-myc transgene) were maintained in DMEM supplemented with 10% FBS and antibiotics. For the serum stimulation experiments, confluent cultures were exposed to 0.25% serum for 3 h. To obtain cells expressed with wild-type c-myc, DMEM containing 10% FBS. Human ATCC 2091 (skin fibroblasts) cells were maintained in DMEM plus 10% FBS. For serum starvation, cells at 70% confluence were switched to DMEM containing 0.1% FBS and incubated for 48 h prior to re-stimulation with serum.

Total RNA was isolated using Trizol (Life Technologies, Inc.). 10 µg of RNA was run on a 1.2% agarose gel, 2 h 143). The total products fell in the linear range of the total input DNA. Relative abundance of each B23 fragment was compared after normalization of the quantity of each fragment in the 2-h ChIP sample to the quantity in the total input DNA sample.

Electrophoretic Mobility Shift Assay—Additional 40-bp double-stranded oligonucleotides, containing the noncanonical wild-type (5’-CACGGG-3’) or mutant (5’-CCCCGGG-3’) boxes most proximal to the B23 transcriptional start site, were synthesized. These oligonucleotides, along with those used in the transactivation experiments, were end-labeled with γ-[32P]ATP and T4 polynucleotide (New England Biosciences) kinase following a standard protocol and purified using Qiagen nucleotide removal kit (Qiagen). For the binding assays, 20-µl reactions were incubated for 20 min at room temperature in 50 mM KCl, 10 mM Tris, pH 8.0, 5% glycerol, 1 mM EDTA, 1 mM DTT with 0.5 µg of labeled oligonucleotides and total protein ranging from 0.5 to 250 ng. Gels were pre-run at 150 V for 1–2 h in 0.5% TBE. The 8% acrylamide gels were loaded with the entire binding reactions and run for ~2 h. Myc-Max heterodimers (gift of Nair and Burley) contain DNA binding domains from both proteins (Myc, amino acids 353–434; Max, amino acids 22–103) and were dialyzed cross-linked. For the competition experiments, 100 ng of protein were incubated with the wild-type probe and either wild-type or double mutant cold oligonucleotide (100 ng to 5 µg).

**Table I**

| ChIP primers                        | Fragment A                  | Fragment B                  | Fragment C                  | Fragment D                  |
|-------------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| B23                                 | 5'-TTTCCCCGGGTTAGAAGGGGC    | 5'-CTGCTCGTGAGATAGATG       | 5'-AGAGCTGGCTTCTGCAAGAAG    | 5'-ATGAGGATCTGTTGTCGCCAG    |
| C23                                 | 5'-GTAGAAGGGACGCGTGGAAG     | 5'-GGGTAAAACGCTAGCTATG      | 5'-GAAGCTGCTCCCGAGAAAC      | 5'-AAACCCAAAGTGGGACTTCC     |
| CAD                                 | 5'-CCGGCTTCTCCGCAGGCGG      | 5'-GTGGGGCCCTGCTATG         | 5'-GACCTGGCTTCTGCAAGAAG     | 5'-GGAGCTGGCTTCACTGACAT     |
| LDH-A                               | 5'-GGGCTCAGGGTGAAGGGGAG     | 5'-GGAGCTGGCTTCTGCAAGAAG    | 5'-GAAGCTGCTCAGAGAGAAGA     | 5'-GGAGCTGGCTTCACTGACAT     |
| Albumin                             | 5'-GGGCTCAGGGTGAAGGGGAG     | 5'-GGAGCTGGCTTCACTGACAT     | 5'-GAAGCTGCTCAGAGAGAAGA     | 5'-GGAGCTGGCTTCACTGACAT     |
| Vimentin                            | 5'-GGGCTCAGGGTGAAGGGGAG     | 5'-GGAGCTGGCTTCACTGACAT     | 5'-GAAGCTGCTCAGAGAGAAGA     | 5'-GGAGCTGGCTTCACTGACAT     |
| Human B23 PCR primers used for ChIP | 5'-GGGCTCAGGGTGAAGGGGAG     | 5'-GGAGCTGGCTTCACTGACAT     | 5'-GAAGCTGCTCAGAGAGAAGA     | 5'-GGAGCTGGCTTCACTGACAT     |

**Rat-specific PCR primers used for ChIP**

| primers                        | Fragment A                  | Fragment B                  | Fragment C                  | Fragment D                  |
|--------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| B23                            | 5'-GTAGAAGGGACGCGTGGAAG     | 5'-TGATAGGGCCGCTGAGG       | 5'-GGGTAAAACGCTAGCTATG      | 5'-AGAGCTGGCTTCTGCAAGAAG    |
| C23                            | 5'-GTAGAAGGGACGCGTGGAAG     | 5'-TGATAGGGCCGCTGAGG       | 5'-GGGTAAAACGCTAGCTATG      | 5'-AGAGCTGGCTTCTGCAAGAAG    |
| CAD                            | 5'-CCGGCTTCTCCGCAGGCGG      | 5'-GTGGGGCCCTGCTATG         | 5'-GACCTGGCTTCTGCAAGAAG     | 5'-GAAGCTGCTCAGAGAGAAGA     |
| LDH-A                          | 5'-GGGCTCAGGGTGAAGGGGAG     | 5'-GGAGCTGGCTTCACTGACAT     | 5'-GAAGCTGCTCAGAGAGAAGA     | 5'-GGAGCTGGCTTCACTGACAT     |
| Albumin                        | 5'-GGGCTCAGGGTGAAGGGGAG     | 5'-GGAGCTGGCTTCACTGACAT     | 5'-GAAGCTGCTCAGAGAGAAGA     | 5'-GGAGCTGGCTTCACTGACAT     |
| Vimentin                       | 5'-GGGCTCAGGGTGAAGGGGAG     | 5'-GGAGCTGGCTTCACTGACAT     | 5'-GAAGCTGCTCAGAGAGAAGA     | 5'-GGAGCTGGCTTCACTGACAT     |
| Human B23 PCR primers used for ChIP | 5'-GGGCTCAGGGTGAAGGGGAG     | 5'-GGAGCTGGCTTCACTGACAT     | 5'-GAAGCTGCTCAGAGAGAAGA     | 5'-GGAGCTGGCTTCACTGACAT     |
A B23 expression in MycER

| Hours | CHX | 4-OHTM | CHX+4-OHTM |
|-------|-----|--------|------------|
| 0     | 0   | 0      | 0          |
| 4     | 1   | 1      | 1          |
| 8     | 2   | 2      | 2          |
| 12    | 3   | 3      | 3          |

Fig. 1. Regulation of B23 expression by c-Myc. A, analysis of B23 mRNA levels in MycER cells after stimulation with 4-OHTM. Hours indicate times when cells were collected after addition of 4-OHTM. Fold induction of B23 was calculated relative to the 0 h-time point. B, Western analysis of B23 and c-myc mRNA levels in TGR and H015 cells after serum stimulation. Vimentin was used as a loading control.

RESULTS

Myc Directly Induces B23 Expression—To identify transcription factors that are directly regulated by Myc, we utilized a previously described (21) Rat1 fibroblast cell line expressing MycER, a fusion protein consisting of Myc and the hormone binding domain of the estrogen receptor. When these cells are exposed to 4-hydroxytamoxifen (4-OHTM), the ligand-bound MycER protein disengages from the cytoplasmic chaperone protein HSP-90 and translocates to the nucleus where it activates or represses transcription of Myc target genes. Because the Myc-ER protein is constitutively expressed, protein synthesis inhibitors do not block its function but do block additional protein synthesis. Incubation of the cells with CHX 30 min prior to exposure with 4-OHTM allows only transcription of direct Myc targets. Suppressive subtractive hybridization was performed on MycER cells incubated for 2 h with either CHX plus 4-OHTM or with CHX alone. B23 was 1 of 12 up-regulated clones detected in this screen. To confirm that B23 is directly induced by c-myc in these cells, two independent MycER inductions were performed and analyzed for B23 mRNA levels. After normalization to the 36B4 (10) loading control, B23 transcript levels increased ~3-fold in cells treated for 8–10 h with either 4-OHTM alone or with 4-OHTM and CHX (Fig. 1A).

We sought to determine whether serum could induce B23 expression in a Myc-dependent fashion. Because c-myc is an immediate early serum-response gene, target genes that are strictly dependent on c-myc should have at least two characteristics. First, target gene expression should follow c-myc expression upon serum stimulation of quiescent cells. Second, expression of the target gene should not be induced in serum-stimulated cells lacking c-myc. Both serum-deprived HO15 (myc −/−) and TGR (myc +/+ ) cells were serum-stimulated over a 24-h period. Total RNA was isolated at various time points and used for Northern blot analysis of B23, c-myc, and vimentin transcript levels. As expected, c-myc transcription was induced within 1-h following stimulation of the TGR (myc +/+ ) cells but was absent in the myc nullizygous cells. B23 mRNA levels began to increase 8 h after serum stimulation of TGR cells and reached levels of 3.5-fold higher than the 0-h time point (Fig. 1B). This induction of the B23 transcript was absent in H015 cells, suggesting that serum induction of B23 expression is Myc-dependent.

Myc Binds to Rat B23 and Other Myc Targets in Vivo—By having observed that B23 may be directly induced by Myc, we sought to determine whether Myc binds the B23 promoter region in vivo. To this end, we employed the ChIP assay with TGR, HO15, and H015 cells stably expressing a c-myc transgene (HO15-myc). This rat fibroblast system has been effectively used with ChIP for the characterization of several known Myc targets (19). Chromatin from log phase cells was immunoprecipitated with antibodies to Myc or E2F4. PCR amplification detected B23, as well as other known c-myc targets (LDH-A, C23, cad), in the TGR and HO15-myc samples but not in the HO15 DNA that were immunoprecipitated with anti-Myc antibody (Fig. 2A). Albumin and vimentin, which are either not expressed or not regulated by Myc, respectively, were not detected in the immunoprecipitate. To ensure that the HO15 chromatin could be immunoprecipitated, we used an anti-E2F4 antibody as a control. Antibodies to E2F proteins, a family of transcription factors that associate with pocket proteins and play an important role in regulation of gene expression at the G1/S phase transition of the cell cycle, have been used previously to determine promoter occupancy of target genes (22). Most promoters analyzed were prominently bound by E2F4. In parallel ChIP assays, both the Myc and E2F4 antibodies precipitated the B23 5′ sequences from TGR cells, and only the E2F4 antibody precipitated B23 sequences in the myc null HO15 cells. By contrast, C23, which does not contain an E2F-binding site in its promoter, was only detected in the anti-Myc antibody-precipitated sample (Fig. 2B).

To substantiate further our observations of Myc-dependent serum stimulation of B23 expression, we performed ChIP with TGR cells that were serum-stimulated for 0, 4, 8, or 16 h. As very little Myc is expressed in serum-deprived fibroblasts, very little B23 and no C23 promoter DNA was detected in the 0-h sample when anti-Myc was used as the precipitating antibody. The quantity of ChIP DNA from both genes increased dramatically at 4 h and remained high to 16 h post-serum stimulation (Fig. 2C). Again, C23 promoter DNA was not detected in any of the anti-E2F4 samples. Interestingly, E2F4 is bound at the B23 promoter at all time points examined. This result is consistent with previous data suggesting that E2F4 is bound to certain promoters from G0 through S phase of the cell cycle (22).

Two Canonical E boxes in Intron 1 of the Human B23 Gene Are Bound by Myc in Vivo and in Vitro and Are Essential for Transactivation by Myc—The emergence of the complete human genome sequence provides a unique opportunity to exploit ChIP for the identification of transcription factor binding sequences. From the human B23 genomic locus, we analyzed 3 kb of sequence, which includes 1 kb upstream of the transcriptional start site, exon 1 and most of intron 1, for Myc binding sites. Two canonical E boxes (5′-CACGTG-3′) were found 10 bp apart in intron 1. Six non-canonical sites also were found, three within 250 bp upstream of the transcriptional start site and three within intron 1 (Fig. 3A). To determine where in this region Myc preferentially binds, we designed PCR primers to amplify ~350-bp fragments to scan the 5′-regulatory and in-
tron 1 B23 sequences by ChIP. Human skin fibroblasts, ATCC 2091, were serum-stimulated for 0 or 2 h. Chromatin was collected, and ChIP analysis was performed using an antibody against Myc (Fig. 3B). Western blot analysis revealed that Myc level is very low at 0 h and peaks at 2 h following serum stimulation in these cells (data not shown). Quantitative analysis using SYBR green and real-time PCR revealed that the fragment containing the two canonical E boxes is significantly more abundant than the other B23-amplified fragments in the 2-h serum-stimulated ChIP sample (Fig. 3C). To ensure that the fragments amplified represent B23 sequences, we sequence-verified all four fragments.

To determine which sequences are important for transcriptional activation of B23 by Myc, we performed reporter assays with luciferase constructs containing sequences from B23 intron 1 or sequences upstream of the transcriptional start site. A reporter construct containing three noncanonical Myc-binding sites from the upstream regulatory region (B23–350) had minimal activity in this assay (Fig. 4A). However, a reporter construct containing 40 bp from intron 1 with the two canonical E boxes (B23 E boxes) exhibited a 4-fold higher activity than the empty plasmid control. This activity is both E box- and Myc-dependent. Mutation of both E boxes from 5′–CAGGTG–3′ to 5′–CCCGGG–3′ (B23 mut) completely abrogates this activity. As expected, a transformation-deficient Myc mutant (Asp106–143) was inactive when assayed with any of our B23 reporter constructs (23).

The three noncanonical Myc-binding sites in the B23 promoter are identical in sequence (5′–CAGGG–3′). Previously, it was shown in vitro that Myc can bind the noncanonical, 5′–CAGGG–3′, and the canonical, 5′–CAGGTG–3′, sequences with similar affinity (24). Our ChIP data, however, suggest that Myc binds more efficiently to the canonical E boxes at the B23 locus in vivo. To evaluate the extent to which Myc binds these sequences in vitro, we performed electrophoretic mobility shift assays using 40 bp double-stranded oligonucleotides containing both the canonical and noncanonical binding sites in the context of the B23 genomic sequence (for oligonucleotide sequences, see Table II). The Myc-Max complex bound an oligonucleotide containing the wild-type E boxes from intron 1 but not an oligonucleotide in which both E boxes have been mutated. This interaction is specific because increasing concentrations of unlabeled wild-type oligonucleotide competed for binding with Myc-Max but the mutant oligonucleotide could not (Fig. 4B). Next, we measured the relative binding of Myc to the
canonical and noncanonical E boxes in B23 by comparing an oligonucleotide containing a single noncanonical binding site most proximal to the transcriptional start site with an oligonucleotide containing two wild type E boxes from intron 1 of B23 or a mutant oligonucleotide. Left 2 lanes represent wild type and mutant oligonucleotides incubated with unlabeled wild type and mutant oligonucleotides, ranging from 100 ng to 5 μg total, were used as competitors in assays with wild type probe and 100 ng total protein. The two complexes represent either one or two E boxes bound. C, relative binding of Myc-Max to a 40-bp oligonucleotide with either one canonical (CACGTG) or one noncanonical (CACGCG) E box. The sequence of the oligonucleotide with the canonical site was derived from B23 intron 1 (the other E box was mutated). The noncanonical oligonucleotide was derived from sequence upstream of the B23 transcriptional start site. Sequences of all oligonucleotides are given in Table II. Oligonucleotides were incubated with increasing amounts of Myc-Max protein. D, graph of percent of each oligonucleotide bound by increasing amounts of Myc-Max protein. The CCCGGG oligonucleotide represents the 40 bp from intron 1 in which both E boxes have been mutated.

**TABLE II**

Sequences of oligonucleotides used in gel shift assays

| Sequences of oligonucleotides used in gel shift assays |
|-------------------------------------------------------|
| Wild-type and mutant-binding sites are capitalized.   |
| 5′ | 10 | 20 | 30 | 3′ |
| cccgctggagCACGTGgttgccACGTGgttggggagcgtggccgggc | B23 intron 1 E boxes (wt E boxes fig 4B) |
| cccgctggagCCCGGGgttgccGGGGgttggggagcggggcggggc | B23 intron 1 double mutant (mut E boxes Fig 4B) |
| cccgaaccggctggagCACGTGgttgccGGGGgttggggagcggggcggggc | B23 intron 1 single E box (CACGTG oligo Fig 4C) |
| gttggagggcttcggagCACGCGcggagcgagcggaggacttg | Wild-type B23 upstream E box (CACGCGG oligo Fig 4C) |
| gttggagggcttcggagCCCGGGcggagcggaggaggcttg | Mutant B23 upstream E box (CCCGGGG oligo Fig 4D) |

**DISCUSSION**

Understanding the biological consequences of transcription requires the identification of genes that are directly regulated by specific transcription factors. Our approach, SchIP, provides a powerful new way not only to verify genes directly regulated by Myc but also to identify precisely sequences important for transcriptional regulation. Until recently, the MycER system was the only tool available to provide evidence that a gene is directly regulated by Myc. Increasingly, chromatin immunoprecipitation is utilized to demonstrate the direct binding of Myc to target genes in vivo thus strengthening the evidence that Myc regulates transcription of these genes (20). Upon identification of B23 as a direct Myc target in the MycER system, we used ChIP with the available rat genomic sequence...
and found that a Myc antibody can precipitate the 5′/H11032 region of the rat gene in wild-type fibroblasts (TGR) but not in c-myc null cells. The previously described c-myc targets cad, LDH-A, and C23, which contain 1, 2, or 4 canonical E boxes in their regulatory regions, respectively, were also precipitated by anti-Myc antibody in this assay. Interestingly, in c-myc null cells ectopically overexpressing Myc, there appears to be an enrichment of these target gene sequences in the ChIP assay over that seen in wild-type cells. We speculate from this observation that elevated Myc protein levels increase the number of target gene sites bound by Myc in a population of cells.

Our use of ChIP has revealed the dynamic association of c-Myc to its genomic targets in the experimental model of serum-stimulated fibroblast growth. Upon serum stimulation of quiescent cells, myc RNA and protein levels increase dramatically within the first 2 h. ChIP analysis of the B23 and C23 promoters revealed that little or no Myc protein is bound in quiescent rat fibroblasts. Association of Myc with B23 and C23 sequences increases significantly by 4 h and remains high until 16 h post-serum stimulation. This correlates well with our observation that steady state B23 mRNA levels begin to increase in these cells 8 h following serum stimulation. A somewhat different result is observed upon serum stimulation of human fibroblasts. In quiescent cells, we detected low levels of Myc binding at both the B23 (Fig. 3) and C23 (data not shown) sequences that greatly increased after 2 h of serum stimulation. The rat fibroblasts are an immortalized cell line whereas the human cells are primary fibroblasts assayed after only 7 passages in culture. Thus, these observations may reflect differences between species of origin or the immortalization state of the cells.

Because the public human genome sequence data base is richer than that of any other mammalian model organism, we utilized ChIP to scan a model human Myc target gene. It is known that Myc can bind to canonical (5′/CACGTG-3′) as well as numerous non-canonical DNA sequences both in vitro (24) and in vivo (25). It is often the case that many putative Myc-binding sites lie throughout a target gene. Complete sequencing of the much smaller yeast genome has facilitated a ChIP approach to locate genomic binding loci of DNA-binding proteins (26). With the nearly complete human genome sequence available, it has become feasible to screen large genomic regions for potential Myc-binding sites. With sequence in hand, it becomes plausible to design ChIP experiments that scan a large region of DNA to identify which sites are bound by Myc. The human B23 gene contains 2 canonical and 6 noncanonical Myc-binding sites in the 3 kb of sequence surrounding the transcriptional start site. Our quantitative scanning ChIP
analysis of this region revealed that the 350-bp sequence containing the 2 E boxes as well as 2 noncanonical sites in intron 1 was enriched 10–20-fold over adjacent sequences of similar size that contain either 1, 3, or no noncanonical sites. Such a strong association, which is corroborated by our gel shift assays and transactivation studies, implies that these sites are important for the transcriptional regulation of B23 by Myc.

We surmise that the combination of human genomic DNA sequence analysis with ChiP is a powerful new approach for studying DNA binding of any transcription factor. Specifically, in the case of Myc, one can begin to discriminate among putative binding sites within a candidate gene to determine which ones are relevant for transcriptional regulation. Our studies with B23 as a model target provide evidence that supports the feasibility of using SChIP. SChIP provides a novel approach to facilitate our understanding of the dynamic binding of transcription factors that regulate the complex circuitry of genes induced under different physiological or pathological conditions.

Acknowledgments—We thank S. Burley, P. Farnham, L. Gardner, L. Lee, S. Nair, L. Resar, and F. Spencer for reagents, protocols, or comments.

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