Transcriptional Control of Nuclear Genes for the Mitochondrial Muscle ADP/ATP Translocator and the ATP Synthase β Subunit

MULTIPLE FACTORS INTERACT WITH THE OXBOX/REBOX PROMOTER SEQUENCES*

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The OXBOX promoter regions of the genes for the muscle-specific adenine nucleotide translocator (ANT1) and the β subunit of the ATP synthase (ATPsynβ) have been implicated in the increased transcription of these nuclear-encoded oxidative phosphorylation (OXPHOS) genes in heart and skeletal muscle. DNA binding, electrophoretic mobility shift (gel-shift) assays now reveal that the OXBOX region has two unique but overlapping elements, the 13-base pair (bp) OXBOX and an 8-bp REBOX. The OXBOX binding factors are found only in myogenic cell lines, whereas the REBOX factors are ubiquitous. Methylation interference experiments have defined the boundaries of the OXBOX and REBOX elements, confirmed that the OXBOX factors are muscle-specific, and shown that the OXBOX and REBOX factors do not bind concurrently. The binding of the REBOX factors was found to be sensitive to NADH and thyroxine, suggesting that it may modulate OXPHOS gene expression in response to environmental and hormonal changes. Hence, the OXBOX/REBOX complex provides one mechanism by which mammalian energy metabolism can be adapted to developmental and environmental demands.

Mitochondria provide most of the ATP for eukaryotic cells through the process of OXPHOS. Five multimeric enzyme complexes are involved in OXPHOS. These are assembled from approximately 100 subunits, 13 encoded by the mitochondrial DNA (mtDNA) and the remainder by the nuclear DNA (nDNA).

Mitochondria of different tissues can differ substantially in their ATP production. Since the mtDNA is relatively uniform in structure and expression among tissues, much of these tissue differences probably result from the differential expression of the nuclear OXPHOS genes.

The heart and skeletal muscle are among the tissues most reliant on mitochondrial ATP production. To determine the molecular mechanism by which this is accomplished, we have studied the transcription of two nDNA OXPHOS genes, ANT1 and ATPsynβ. The mRNA levels of both of these genes are very high in heart and muscle, and the expression is generally coordinated with that of the mtDNA (Webster et al., 1990; Torroni et al., 1991; and Stepien et al., 1992).

Comparison of the promoters of ANT1 and ATPsynβ revealed that they shared a 13-bp1 promoter element, the OXBOX, present in the same orientation and 0.45 kilobases (kb) upstream from the putative transcription start sites (Li et al., 1989; Neckelmann et al., 1989). The OXBOX was shown to be a muscle-specific positive transcriptional element (Li et al., 1990), thus accounting for the coordinate induction of these genes in muscle.

To further characterize the muscle regulation of these genes, we have performed extensive gel-shift assays in the OXBOX region. These experiments have revealed a new sequence element, the REBOX, overlapping the OXBOX in both ANT1 and ATPsynβ promoters. This REBOX binds sequence-specific factors that are present in human HeLa and myogenic mouse C2C12 (Yaffe and Saxel, 1977) and Sol8 (Mulle et al., 1988) cells. However, OXBOX binding factors are found only in the myogenic cell lines.

MATERIALS AND METHODS

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1 The abbreviations used are: bp, base pair(s); T3, triiodothyronine; DTT, dithiothreitol; ds, double strand.
The excised methylated DNAs were cleaved with 100 mM piperidine at 95 °C for 30 min. All samples were desiccated and resuspended in glass-distilled deionized water. 2000 cpm of sample were run on a denaturing 8% polyacrylamide gel (6 M urea, 20:1 acrylamide/bisacrylamide ratio, 1 × TBE). The gel was dried on Whatman paper and exposed to film for 7–14 days with enhancement screens.

**Quantitative Determination and Analysis**—Radiographs from interference experiments were scanned with a Pharmacia laser densitometer, and raw optical density values were normalized against the uncleaved probe band at the top of each lane. These normalized values were compared with the two flanking “free” probe lanes (see Fig. 8 and 9, side lanes). The percentage values of either attenuation or enhancement were graphically incorporated (see Table II).

**RESULTS**

Five different 40-bp dsDNA oligomers (40 mers) were used to examine protein-DNA interactions in the OXBOX/REBOX region of ANT1 and ATPsynβ. Three of these were derived from the wild-type ANT1-OXBOX sequence (Li et al., 1990) and are designated mut1, 2, and 3. The β-OB 40-mer contains the wild-type ATPsynβ OXBOX region. The numbering scheme starts at the 5’-end of the 40 mers with the same orientation as the published promoter sequences (Fig. 1) (Li et al., 1989; Neckelmann et al., 1989).

In ANT1-OB, the OXBOX element spans the 13-bp region G-14 to G-26, while the REBOX element is an octamer from G-26 to T-33. In β-OB, the OXBOX is G-14 to G-26, but the REBOX is C-28 to A-21. The mut1 40-mer replaces the ANT1-OB C-16 and G-25 with transversions A-16 and T-25, thus conserving the dyad symmetry. The mut2 40-mer changes the ANT1-OB OXBOX at all 13-nucleotide pair positions and also changes G-26 to A-26 in the REBOX. The mut3 40-mer of ANT1-OB alters only the REBOX, substituting C-28 with A-28.

**Mobility Shift Gel Electrophoresis Revealed Factors That Recognize Two Overlapping Sequence Elements**—Mobility shift gel electrophoresis (gel-shift) experiments with C2C12 nuclear extracts revealed four sequence-specific complexes: α, β, A, and B (Fig. 2). The “B-band” had previously been observed and proposed to represent a complex involving the OXBOX element (Li et al., 1990). However, further analysis has shown that it represents factor binding to a flanking sequence. When mut1 and mut2 were used as probes, the B-band complex was still present and this band was competed away by cold ANT1-OB, mut1, and mut2 40-mers (Fig. 2, lanes 7–17) but not by random oligomer sequences (not shown).

Gel-shift analysis using mut3, with a single base change 3′ to the OXBOX (C28 → A), mapped the B-band DNA element to the sequences immediately downstream from the OXBOX (Fig. 4, lane 4). This was confirmed in competition assays where the mut3 40-mer was the only cold competitor that was unable to attenuate the B-band signal (not shown). Thus, C-28 is critical for recognition by the B-band factor.

Gel-shift with C2C12 nuclear extracts and the β-OB probe revealed that the ATPsynβ-derived 40-mer also produces a B-band (Fig. 3, lanes 1 and 6, Fig. 7, lanes C and D). The

**Fig. 1. Sequences used in gel-shift assays.** OXBOX and REBOX elements are boxed. Point mutations are marked by arrows.

**Fig. 2. Gel-shift assays demonstrating sequence specificity.** Autoradiographs of dried native polyacrylamide gels with three different 32P-labeled 40-mer dsDNA probes, ANT1-OB, mut1, and mut2. C2C12 nuclear extract was used in each binding reaction. The amount of cold competitor in each lane (0, 10, or 20 ng) also included 40 ng of poly(dI-dC).

**Fig. 3. Gel-shift assays using ATPsynβ probe.** Autoradiographs of dried native polyacrylamide gels with three different 32P-labeled 40-mer dsDNA probes, ANT1-OB, mut1, and mut2. C2C12 nuclear extract was used in each binding reaction with 20 ng of the indicated cold competitor (OB, Mut1, Mut2, or βOB) in addition to 40 ng of poly(dI-dC). Lanes 1 and 6 only have poly(dI-dC) competitor.

**Factors binding to β-OB are likely to be the same as OB, mut1, and mut2 for three reasons. First, they approximately co-migrate and exhibit identical behavior in the presence of different competitor sequences. Cold OB, mut1, mut2, and βOB 40-mers are equally effective at attenuating the B-band signal irrespective of the probe used (Figs. 2, 3, and 5, lanes 13–17, and other data not shown). Second, all B-band complexes have a sharp pH optimum at 7.6 with 0.2 pH unit changes in either direction being sufficient to reduce the B-band complex (not shown). Third, all B-band complexes, irrespective of probe involved, exhibited an unusual DTT-dependent binding, strong at 0 mM DTT, eliminated at 1 mM, and renewed at 4 mM (Fig. 5). Since ANT1-OB and β-OB yield the same complex, they must share a “consensus” sequence element other than the OXBOX. The only region of
the β-OB 40-mer that is similar to the area surrounding C-28 in ANTI-OB is from C-28 to A-21 on the complementary strand of the β-OB dsDNA oligomer. The REBOX consensus element must be contained within the octamer (5′-(Pu)CCC(A/T)Py(A/T)T′-3′) shared by OB, mut1, mut2, and βOB.

The more slowly migrating α and β bands contain factor(s) that recognize the ANTI-OB OXBOX element (Fig. 2). These complexes are more labile than the B-band and require that gel electrophoresis be performed at 4 °C rather than at the room temperature adequate for the B-band (Li et al., 1990). Their specificity for the OXBOX sequence was confirmed by using either mut1 or mut2 as probes (Fig. 2). Mut2 lacks the OXBOX and mut1 has two internal transversions A-16 and T-25 (Fig. 1).

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Multiple OXBOX/REBOX Transcription Factors

concentration, remaining missing when DTT was absent and appearing only when DTT was present. These results imply that the REBOX factors bind if the redox potential is either low or high but not moderate.

Thyroid hormone at micromolar levels specifically inhibits B-band complex formation and alters the mobility of α and β complexes (Fig. 6). This effect was seen for both T3 (lanes 4–6) and T4 (lanes 7–9) but not for T2 (lane 11). Moreover, T3 (lanes 5, 6, and 10) but not T4 (lanes 7–9) causes the formation of larger complexes that do not enter the gel (Fig. 6). These results suggest that thyroid hormone can interact with OXBOX/REBOX complexes and affect their binding and aggregation.

Methylation Interference of OXBOX and REBOX Binding Sites—The G residues which are important for OXBOX and REBOX binding were identified by methylation interference. The ANT1-OB and ATPsynβ probes were 5'-end-labeled on each strand (me-OB and me-cOB for ANT1-OB and me-βOB and me-βOB for ATPsynβ) and dimethyl sulfate-methylated, and the bands were excised from preparative gel-shift assays (Fig. 7). The methylated me-OB and me-cOB were incubated with C2C12, So18, and HeLa extracts, while the methylated me-βOB and me-βOB were incubated with C2C12 extracts. The preparative gels for the C2C12 extract is shown in Fig. 5.

**Fig. 5.** Gel-shift assay with varying concentrations of DTT. Autoradiograph of a dried native polyacrylamide gel of 32P-labeled ANT1-OB probe. Each lane included 40 ng of poly(dT-dC) in addition to 20 ng of the indicated cold competitor.

**Fig. 6.** Gel-shift assay with varying levels of thyroid hormone. Autoradiograph of a dried native polyacrylamide gel of 32P-labeled ANT1-OB probe. Each lane includes 40 ng of poly(dT-dC) in addition to 20 ng of cold mut2 competitor in lane 4.

**Fig. 7.** Large scale gel-shift assay with dimethyl sulfatemethylated probes. Autoradiograph of a wet native polyacrylamide gel of partially methylated 32P-labeled 40-mer dsDNA probes incubated with C2C12 extract. Partially methylated ANT1-OB, 5'-end-labeled on the sense-strand is designated me-OB. Similarly, ANT1-OB, labeled on the complementary strand, is designated me-cOB. The β-OB-derived probes are analogously designated me-βOB and me-βOB. Bands were subsequently excised, purified, and cleaved with piperidine to yield fragments displayed in Figs. 8 and 9.

**Fig. 8.** Methylation interference of the binding of C2C12 extract to the ANT1-OB probe. Partially methylated ANT1-OB probe was extracted from the bands a, b, A, and B that were excised from the preparative gel shown in Fig. 7 (lanes A and B). The DNAs were purified, cleaved with piperidine, and separated on a denaturing polyacrylamide gel. The gel was dried and the autoradiograph is shown. Cleavage patterns for the me-OB probe (Fig. 7, lane A) are free probe (lanes 1 and 6), a (lane 2), b (lane 3), A (lane 4), and B (lane 5). Patterns for the me-cOB probe (Fig. 7, lane B) are free probe (lanes 7 and 12), a (lane 8), b (lane 9), A (lane 10), and B (lane 11). The location of cleaved G residues are mapped on the sides.

7. The corresponding interference patterns are shown in Figs. 8 and 9 and summarized along with those of So18 and HeLa in Table II. For clarity, please refer to Table II for all subsequent references to methylation interference positions.

The ANT1-OB DNA-protein complexes in the B-band lacked methylation at G-26, C-27, C-28, and C-29, irrespective of the extract used (Table II). Similar βOB probe interference occurs at C-28, G-27, G-26, and G-23, while the methylation of G-25 is enhanced (Fig. 9, lanes B, sense and antisense). Enhancement is sometimes seen in methylation interference
Table II
Summary of methylation interference data

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assays, and its implications concerning the DNA-protein interactions will be covered later (see “Discussion”).

The complexes in the OXBOX region giving the α, β, β1, and β2 bands were more heterogeneous. For the ANT1-OB probe, the interference patterns of muscle cell extracts were remarkably similar for the α- and β-bands (Table II). In the α-bands, weak contacts (i.e., moderate interference) were made at G-14, G-15, C-16, C-18, and strong contacts (i.e., higher interference) were made at G-23, G-25, and G-26 (Fig. 8, lanes α, sense and antisense, and Table II). In the β-bands, the contact at G-14 is stronger than in the α-bands, but the G-15, G-16, C-18, and C-19 contacts are the same (Table II). Moreover, in Sol8 extracts, the β-band binding factor(s) also makes a strong contact at C-16. The larger number of strong contacts in the myogenic β-band within the OXBOX element further supports the gel-shift evidence that this subset of OXBOX binding factor(s) may have more stringent OXBOX sequence requirements (Fig. 2, compare lanes 4 and 5) than myogenic α-band factor(s). The β-like band associated with HeLa extract in-12.8 extracts involves factor(s) that make light contacts at G-14, G-15, and G-26 and heavy contacts at C-18 and C-23 (Table II). This clearly distinguishes the HeLa factor(s) and is additional evidence that HeLa cells lack factors that recognize the OXBOX control element. Since the HeLa β-like band is not lost by using either the Mut2 or Mut3 probes, neither the OXBOX contacts nor the C-28 REBOX contact are involved in sequence recognition by the HeLa factor(s).

For the ATPsynβ-derived methylated probes, the contacts of OXBOX binding factor(s) occur at nucleotides G-14, G-15, C-16, and C-18, the upstream half-site of the OXBOX element rather than the downstream half-site as was evident for the methylated ANT1-OB probes (Fig. 9, lanes β1 and β2, sense and antisense). Moreover, the interference patterns for the β-OB β1 and β2 complexes are more different from each other than was the case for the ANT1-OB α and β complexes. In the β1 OXBOX binding factor-DNA complexes, weak major groove contacts were made at G-23 and G-26, while strong contacts were made at G-14, G-15, C-16, and C-18 (Fig. 9, lanes β1, sense and antisense). In the β2 complexes, there is no contact at G-23 and instead of a strong contact at C-18 there is some enhancement (Fig. 9, compare lanes β2 with β1 at C-18 on the antisense strand). None of the complexes have interference patterns that would suggest the simultaneous binding of both OXBOX binding factors and REBOX binding factors.

The differences between the OXBOX contacts of ANT1-OB and β-OB probes might be explained in two ways. The factors that recognize the ANT1-OXBOX and those that recognize the ATPsynβ-OXBOX may be distinct and regulate these genes differently. Alternatively, the factors may be the same but the OXBOX sites might be different due to the effects of flanking sequences. The latter is supported by the gel-shift experiments with competition oligomers (Fig. 5, compare lanes 14 and 15 with 16 and 17) and could be explained if the symmetrical half-sites of the OXBOX together bound a dimeric molecule whose monomers would bind more strongly to the half-site with the most optimal flanking sequence.

REBOX Binding Factors May Bind DNA—Subtle differences were noted in the mobility of B-band complexes with the ANT1-OB and β-OB probes (Fig. 7, compare lanes B with C), where the β-OB probe B-band complex was consistently faster. The mobility of the B-band complex was also distinctly faster for the ANT1-OB probe than for the mut2 probe (Fig. 4, compare lanes 2 with 3, lanes 5 with 6, and lanes 8 with 9). Since the mut2 probe has an intact REBOX, but has all of the OXBOX nucleotides substituted, this phenomenon might be explained in two ways. The REBOX binding factor(s) may assume different conformational states depending on the adjacent OXBOX sequences similar to those observed for GCN4-DNA complexes (Gartenberg et al., 1990). Alternatively, the REBOX binding factors might bend the DNA upstream from the REBOX similar to DNA bending that has been reported for the canonical zinc-finger protein TFIIIA (Schroth et al., 1991, 1989) and the zinc-finger protein SP1 (Morgan et al., 1988) upon sequence specific binding.
The current gel-shifts required zinc cations, suggesting that some of the factors may be zinc-finger proteins. Since zinc-finger proteins are associated with DNA-bending (Schröth et al., 1991), we hypothesize that bent DNA might explain the anomalous migration of REBOX complexes. The mut2 complex would then migrate differently because the higher A/T content of the mutated OXBOX region (9 A/T pairs versus 6) would alter the degree of bending. Similarly, the REBOX binding factor-βOB complex would have the highest mobility, because the inverted orientation of the REBOX would place the bend at the 3'-end of the 40-mer, whereas the REBOX binding factor-OB and REBOX binding factor-mut2 complexes would be slower since their bends would be in the middle (as illustrated in Fig. 10).

DNA bending by the REBOX-protein complex is supported by the DNA methylation interference experiments (Table II). The band β REBOX complexes showed strong methylation interference at C-16 for C2C12 extracts even though mutations at this position (A-16 in mut1 and G-16 in mut2) have no effect on complex formation. Likewise, strong methylation interference is seen at the C-18 for band B REBOX complex formation in HeLa, and weaker methylation interference is seen at G-14, G-15, C-16, and C-18 in band B for Sol9. This, REBOX binding factors appear to make contacts approxi-mately one helical turn (10 bp) upstream from its recognition site. These upstream contacts which are not required for factor binding could be consistent with the hypothesis that REBOX binding factor(s) are inducing a bend upstream from the REBOX.

**DISCUSSION**

Striated muscle is one of the most oxidative types of mammalian tissue. When contractile work is being performed, the actinomyosin ATPase in fast-twitch oxidative fibers causes the tissue to consume 350 µmol/min of ATP per g wet weight (Hochachka et al., 1991). This far exceeds the ATP turnover rates in “housekeeping” tissues such as intestine, liver, and kidney. Such demands on OXPHOS are met in two ways. First, there is the observed elaboration of a number of muscle-specific isoforms of nDNA-encoded OXPHOS genes (Schultheiss and Klingenberg, 1984; Schlerf et al., 1988; Yamamura et al., 1988; Kadenbach et al., 1990; Gay and Walker, 1985; Haas and Strauss, 1990). Second, there are increased levels of OXPHOS gene transcripts for both muscle-specific isoforms and housekeeping genes such as ATPsynβ, cytochrome c1, and mtDNA-encoded genes (Neckelmann et al., 1989; Webster et al., 1990; Suzuki et al., 1989; Stepien et al., 1992).

The OXPHOS capacity of muscle to perform work may also be subject to short-term adaptive controls. With sustained electrically stimulated contractions, the transcript levels of OXPHOS genes increase as the levels of glycolytic genes decrease (Williams et al., 1986; Williams et al., 1987). Moreover, a switch from low to normal oxygen tension during the transition of cultured myoblasts to myotubes induces OXPHOS gene transcript levels (Webster et al., 1990). Accordingly, muscle-specific OXPHOS gene regulation must involve both tissue-specific and environmentally modulated cis transcriptional elements. The current data indicate that these include the OXBOX, a positive muscle element, and the REBOX, a redox-sensitive (possibly negative) control element.

The muscle-specific expression of ANti and ATPsynβ is clearly mediated through the α and β protein complexes interacting with the OXBOX. Previous transient transfection studies (Li et al., 1990) demonstrated that changes in the OXBOX element decreased the expression of ANti promoter-reporter gene constructs in C2C12 myoblasts (Li et al., 1990), and the current gel-shift experiments have shown that the same OXBOX ablation mutants (Mut2) eliminates the α and β band complexes.

Moreover, the current experiments have shown that the various OXBOX binding factors are found exclusively in myogenic cells, not in HeLa. Transient transfection assays have shown that ANti reporter gene activity was low in HeLa cells (Li et al., 1990), a result consistent with low ANti and ATPsynβ mRNA levels in HeLa cells (Torroni et al., 1990; Lunardi and Attardi, 1991; Neckelmann et al., 1989).

The dyad symmetry of the OXBOX element and the differences in OXBOX methylation interference between ANTI-OB and β-OB suggest that OXBOX binding factors bind as a dimer (Fig. 10) and that flanking sequences affect the binding efficiency. Such a model would be similar to the proto-oncogene product c-Jun, which dimerizes with either itself or c-Fos to form molecules with different binding affinities to the symmetrical AP1 cis element (Halazonetis et al., 1988). Other examples of transcription factors that bind as dimers to sites with dyad symmetry include AP2 (Williams and Tjian, 1991), AP-4 (Hu et al., 1990), CREB (Yun et al., 1990), USF (Gregor et al., 1990), MyoD (Weintraub et al., 1991), E2 (Moskaluk and Bastia, 1989), BZLF1 (Kouzarides et al., 1991), GCN4 (Hope and Struhl, 1987), and LAC9 (Halvorsen et al., 1991). The general trend is that cis elements with 2-fold symmetry are bound by dimeric protein complexes (for a review see Jones, 1990).

The discovery of the overlapping REBOX element and its binding factor adds a redox-sensitive, possibly negative element to the system. This could modulate transcript levels according to surrounding oxygen tension. Factors that bind the REBOX are present in HeLa cells, where the ANTI gene is essentially “off.” This and its putative DNA-bend-
erties (Fig. 10) suggest that the REBOX binding factor(s) may have some features in common with certain prokaryotic transcriptional repressors such as bacteriophage 434 repressor (Koudelka et al., 1988), Tet repressor (Tovar and Hillen, 1989), LexA repressor (Lloubes et al., 1988), Gal and Lac repressors (Zwieb et al., 1989).

The competitive interactions between factors for binding to the OXBOX/REBOX region is demonstrated in vitro by the methylation “enhancement” in the interference experiments. These enhancement signals likely indicate where a methylation event favors the binding of a given factor by preventing the binding of a competing factor. Methylation enhancement was seen in the URE/CRE region of the glucocorticoid hormone α gene, where there was evidence that two URE domains overlapped and steric hindrances prevented simultaneous occupancy (Jamieson et al., 1989). For the ANTI-OB probe bound to C2C12 extract, methylation enhancements at C-34 and C-35 for the A and B bands imply that the methylation event favors the binding of A- and B-band factors, while preventing the binding of competing β-band factors as indicated by methylation interference at C-34 and C-35 (Table II). Similarly, for the β-OB probe bound to C2C12 extract, methylation enhancement at C-16 and C-18 for the A and B bands versus interference at C-16 for β and β2 and C-18 for β1 is additional evidence for the mutually exclusive nature of the REBOX and OXBOX factor binding (Fig. 9, lanes A and B on the antisense strand and Table II).

The remarkable similarity of the ANTI-OB interference patterns in the area of the REBOX (G-26, C-27, C-28, and C-29) for all myogenic extracts demonstrates that all factors bind in the major groove of this region and recognize the overlapping OXBOX/REBOX elements (Fig. 8, sense and antisense strands, and Table II). However, it is clear that only the binding of the REBOX B-band requires a specific nucleotide sequence in this area since only the B complex is affected by the C-28 mutation (Fig. 4, lanes 4, 7, and 10). Although methylation at ANTI-OB G-26 interferes with the formation of B-band complexes, a G to A change at this base did not ablate REBOX factor binding. This suggests that like footprinting techniques, interference patterns effectively reveal the location of binding, but not necessarily the location of sequence recognition. A similar conclusion was reached for XFI, XF2, and Ah factor binding to a promoter region in the cytomegalo PDGα gene, where the three distinct factors exhibited near identical methylation interference patterns (Saatcioglu et al., 1990).

The interference data in this study demonstrate that OXBOX/REBOX factor binding involves a complicated interplay of trans-acting elements. Methylation interference at G-26, C-27, C-28, and C-29 of the ANTI-OB probe for α, β, A, and B-bands supports the existence of steric constraints that would prevent all four complexes from coexisting simultaneously (Fig. 8 and Table II). This mutually exclusive property of OXBOX/REBOX factor binding is revealed by the distinctness of each of the protein-DNA complexes as elucidated by the gel-shift data (Figs. 2–4). Methylation interference patterns on the β-OB probe with C2C12 extract further support that OXBOX and REBOX factor binding antagonize each other. No β-OB band produces interference at both the OXBOX and REBOX (Table II).

REBOX binding factor-DNA complex formation is exquisitely sensitive to pH, DTT, NAH, and Tr concentrations (Figs. 5 and 6 and other data not shown). This raises the possibility that REBOX binding factor(s) are a “molecular sensor” of cellular energy requirements, “measuring” pH, redox state, and thyroxine levels of the cell. DTT is a potent reducing agent, whereas NAH is a less potent one with greater physiological importance. Recent studies have shown that the concentration of NAH in striated muscle is a good cytosolic indicator of how well the tissue’s production of ATP by OXPHOS matches consumption (Duboc et al., 1988; Duboc et al., 1990; Esumi et al., 1991), and thyroxine has been implicated in several aspects of mitochondrial biogenesis and OXPHOS function at the level of transcriptional initiation (Mutvei et al., 1988; Joste et al., 1989).

The much higher than physiological levels of thyroxine needed to observe the specific inhibition of REBOX DNA-protein complexes may simply reflect the nonphysiological conditions of the in vitro gel-shift assay. Perhaps, with the proper physiological concentrations of all as yet undetermined factors that affect REBOX factor binding, even low physiological concentrations of thyroxine may clearly demonstrate specific inhibition.

The REBOX binding factor’s responsiveness to environmental stimuli and its manifold interactions with trans-activating OXBOX binding factors is reminiscent of α-fetoprotein gene regulation by interactions between AP-1 and the glucocorticoid receptor (Zhang et al., 1991a). Indeed, REBOX binding factor(s), owing to its specific sensitivity to thyroxine, may be another member of the steroid hormone receptor family along with thyroid and glucocorticoid hormone receptors. It has been observed that although steroid hormone receptors primarily act as trans-activators, they can also repress transcription (Zhang et al., 1991a; Zhang et al., 1991b; Koenig et al., 1989; Damm et al., 1989; Graupner et al., 1989). Accordingly, the REBOX binding factor(s) may silence transcription either by antagonizing the binding of the OXBOX trans-activator or by intrinsically inhibiting transcription complex formation or both. This model of OXBOX activation counter-balanced with REBOX repression harkens back to the “push-pull mechanism” of gene regulation seen in the steroid-dependent repression of the LDL receptor promoter against SP1 activation (Dawson et al., 1988) and the competitive interactions between Zif268 and SP1 for binding to a cis-acting “repressor” element and an SP1 site, respectively (Ackerman et al., 1991).

Moreover, the OXBOX and REBOX binding factors join the Mt binding factors (Suzuki et al., 1991) and NRF-1 (Evans and Scarpulla, 1989, 1989) as regulatory factors that may be involved in coordinating the expression of nDNA-encoded OXPHOS genes. Since OXBOX binding factors are exclusively found in myogenic cells, perhaps they are part of a distinct muscle-specific regulatory system. On the other hand, the REBOX binding factor(s) appears to be ubiquitous, likely active in most tissues and may be a molecular sensor that coordinates the expression of OXPHOS genes to match cellular energy requirements.

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REFERENCES


