OXBOX, a Positive Transcriptional Element of the Heart-Skeletal Muscle ADP/ATP Translocator Gene*

Kang Li, Judy A. Hodge, and Douglas C. Wallace

From the Departments of Biochemistry, Pediatrics, and Neurology, Emory University School of Medicine, Atlanta, Georgia 30322

Three positive transcriptional control regions have been identified in the promoter of the human heart-skeletal muscle adenine nucleotide translocator gene (ANT1). By transfecting promoter-chloramphenicol acetyltransferase fusion constructs into C2C12 myogenic cells, each positive region was found to increase transcription 2-3-fold. The first region spans from -123 to -674 base pairs (bp), the second from -2.6 to -3.1 kilobases, and the third from -3.1 to -8.8 kilobases. Linker-scanning mutants generated using the polymerase chain reaction and modified oligonucleotides have identified the OXBOX (5’-GGCTCTAAA-GAGG) as the positive element within the -123 to -674-bp region. This element enhances transcription in muscle cells but not in HeLa cells, suggesting that it is muscle-specific. Gel retardation experiments have revealed a factor from C2C12 cells which specifically binds to a 40-bp piece of the ANTI promoter containing the OXBOX. Since the OXBOX is also found in the promoter of the human ATP synthase β subunit gene, it is the first tissue-specific element identified which could coordinately regulate mitochondrial oxidative phosphorylation genes.

The adenine nucleotide (ADP/ATP) translocator (ANT) is a homodimeric protein of the mitochondrial inner membrane (1, 2) which transports ADP into and ATP out of the mitochondrion. In eukaryotic cells, mitochondrial ATP production and cellular ATP consumption are linked through ANT, the kinetics of which have been proposed to play a major regulatory role in mitochondrial OXPHOS (3-7).

Multiple ANT isoforms have been identified. These were initially recognized by antigenic and electrophoretic differences among heart, liver, and kidney ANTs (8, 9) and subsequently confirmed by the isolation and characterization of three human and two bovine ANT cDNAs. We have characterized a complete human ANT cDNA (ANTI) (10), which is expressed primarily in heart and skeletal muscle (11). A second human cDNA has been isolated from fibroblast cells (hp2Fl) or ANT2 (12) and found to be expressed at significantly lower levels in heart, skeletal muscle, liver, kidney, and brain (11). A third human cDNA has been isolated from liver (pHAT8 or ANT3) (13). The two published bovine cDNAs are homologues of ANTI and ANT3 (14).

Recently, we have cloned and sequenced the human ANTI gene (11). Comparison with the human ANTI (T2) gene (15) revealed that the intron-exon structure of the two genes is the same, but that their gene regulatory sequences are markedly different. Classical TATA and CCAAT boxes are present in the ANTI gene, but not in the ANT3 gene. Both of the genes have Sp1 binding motifs, GC boxes, in the first intron. However, the ANT3 gene has 12 additional GC boxes in the promoter region, whereas the ANTI gene lacks these.

Comparison of the ANTI and ATP synthase β subunit (ATPsynβ) promoters revealed a 13-bp homologous sequence, the OXBOX (11, 16, 17). This sequence is located at a similar position in these genes, but is not found in ANT3 (15). We have postulated that the OXBOX might be a cis-acting element regulating transcription of the ANTI and ATPsynβ genes (11, 17).

To identify the transcriptional regulatory elements of the ANTI promoter and to determine if the OXBOX is a transcriptional element, we prepared a series of promoter-reporter gene constructs using chloramphenicol acetyltransferase. The expression of these constructs was analyzed in mouse myogenic C2C12 cells (18) and HeLa cells, and three major positive muscle regulatory regions were mapped, one of which was identified as the OXBOX.

MATERIALS AND METHODS

Preparation of Promoter Deletions Using Restriction Fragments—ANTI promoter deletions were constructed by polymerase chain reaction (PCR) amplification of the region from the EcoRI site at -674 bp to the middle of the 5’-nontranslated region at +42 bp. The downstream PCR oligonucleotide primer (Emory University Microchemical Facility for Molecular Biology) included a BamHI restriction site at the 5’-end for subsequent cloning. This fragment was cloned into plasmid pRLCAT8, a promoter-less CAT plasmid provided by Bruno Luckow, Heidelberg, Federal Republic of Germany (19). Additional promoter elements were added by ligation of upstream restriction fragments (Fig. 1) (11). To test the transcriptional role of the Sp1 sites in the first intron, virtually the entire first intron (1.2 kb) was PCR-amplified, and the products were cloned into the KpnI site 3’ to the CAT reporter gene in both orientations (pBLANTCAT5b and pBLANTCAT5c; Fig. 1).

PCR Linker Ablation and Construction of Chimeric Plasmids—The OXBOX was ablated by PCR amplification of the surrounding region using two pairs of oligonucleotides which overlapped at the OXBOX and substituting a BglII site for it (Fig. 2). Primers 2 and 4 amplified a 556-bp fragment between the OXBOX and +42 bp, and primers 1 and 3 amplified a 205-bp fragment between the OXBOX and -674 bp. The PCR template was a HindIII-linearized subclone of the ANTI promoter plasmid pHANT1/r. PCRs were performed using a thermal cycler (Perkin-Elmer) and Taq DNA polymerase (Perkin-Elmer Cetus). The two fragments were joined at the BglII site and subcloned.

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**The abbreviations used are: ANT, adenine nucleotide (ADP/ATP) translocator; OXPHOS, oxidative phosphorylation; bp, base pair(s); kb, kilobase(s); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CAT, chloramphenicol acetyltransferase; ATPsynβ, ATP synthase β subunit; PCR, polymerase chain reaction.
into a Bluescript vector. Ablation of the OXBOX was confirmed by deoxy chain termination sequencing (21) and purified through two cycles of CsCl/ethidium bromide gradient ultracentrifugation. The DNA concentration was measured by UV spectrophotometry.

**Transient Transfection by Calcium Phosphate Precipitation**—The mouse C2C12 myoblast cells were maintained in Dulbecco's modified Eagle's medium with 20% fetal calf serum (HyClone Laboratories, Logan, UT). Cultures were inoculated at 8 × 10⁵ cells/100-mm dish, and the medium was changed the next day. Two to four hours later, 25-50 μg of calcium phosphate precipitated supercoiled plasmid (22) was added to the medium. The DNA precipitate was prepared by adding 0.5 ml of plasmid in 250 mM CaCl₂ to 0.5 ml of 9 × HBS (250 mM NaCl, 50 mM HEPES, 1.5 mM Na₂HPO₄, pH 7.09) with constant mixing by aeration and then standing at room temperature for about 30 min. After 17-20 h, the medium was changed, and the cultures were grown for another 24 h. Half of the cultures were further stimulated to form myotubes by switching to 2% horse serum in Dulbeco's modified Eagle's medium and growing for an additional 48 h. HeLa cells were grown in modified Eagle's medium with 10% fetal calf serum and transfected with 30 μg of each plasmid. Four and one-half hours after the DNA was added, the cells were rinsed in modified Eagle's medium and exposed to 15% glycerol (v/v) in 1 × HBS at room temperature for 3 min. The culture was washed twice in phosphate-buffered saline and incubated for an additional 42 h in fresh medium.

**Chloramphenicol Acetyltransferase Assay**—Cells were rinsed twice in phosphate-buffered saline and harvested using 1.4 ml of 1 M EDTA, 50 mM Tris, pH 7.5/plate. Cell pellets were suspended in 100 μl of 250 mM Tris, pH 7.5, and duplicates of each plasmid transfection were mixed. Half of the mixture was used to quantify plasmid DNA by Hirt (23) extraction. The other half was assayed for CAT activity following lysis using three freeze-thaw cycles (22). Five or ten microliters of C2C12 cell lysate or 20 μl of HeLa cell lysate was included in a 150-μl reaction containing 0.2 μCi of [³⁵S] chloramphenicol, 0.5 mM acetyl-CoA, and 250 mM Tris, pH 7.5. After 20 min at 37°C, chloramphenicol was extracted in ethyl acetate and lyophilized. Acetylated and nonacetylated chloramphenicol were separated by thin-layer chromatography, and the spots were excised and quantified by liquid scintillation counting.

**Quantitation of Transfected Plasmids by Hirt** (23) Extraction—Multiple plasmid constructs were tested in each experiment. To normalize the CAT activity between transfections within each experiment, the percent acetylated chloramphenicol for each transfection was corrected by the amount of plasmid taken up by the cells. Plasmid DNA was enriched from transfected cells by salt precipitation of chromatin (23). The resulting DNA was digested with EcoRI, separated on an 0.8% agarose gel, transferred to a Millipore nylon membrane, and hybridized with a 32P-labeled random primer-synthesized (24) vector DNA probe. The bands detected by autoradiography were cut out of the filter and counted. The percentages of chloramphenicol acetylated for each plasmid within an experiment were normalized by expressing the results as a percentage of the values obtained for plasmids pBLfOB+CAT and pBLfOB−CAT for C2C12 cells and pSV5/CAT for HeLa cells.

**Gel Mobility Shift Assay**—Protein-OXBOX complexes were detected by using a 40-bp AN1T promoter probe encompassing the OXBOX. This probe was prepared by annealing two complementary oligonucleotides (5'-CTCGGAGGAGGACCCATCTAAAAGG-3' OXBOX underlined) (11). The oligonucleotides were labeled with [³²P]ATP and T kinase and annealed in 150 mM NaCl, 1 mM ZnCl₂, and the double-stranded probe was purified by electrophoresis on a 6% polyacrylamide gel. C2C12 nuclear extract was prepared following the method of Dignam et al. (25).

**OXBOX Probe and nuclear extract were incubated at 30°C for 15 min in 40 mM HEPES, 60 mM KCl, 0.5 mM ZnCl₂, and the double-stranded probe was purified by electrophoresis on a 6% polyacrylamide gel running in circulating low ionic strength buffer (7 mM Tris, 3.5 mM NaCl, 1 mM EDTA, pH 7.5) (26). The gel was dried onto Whatman paper and exposed to Kodak autoradiograph film.

The specificity of binding was demonstrated by competition with unlabeled probe (cold probe) and with a nonspecific probe with the sequence 5'-GATCTCTTGTGTCCTGGACCCTTTGCAAGAA-3'. Competitor oligonucleotides were added together with the labeled probe during the binding reaction.

**RESULTS**

Identification of Multiple cis-Acting Elements in ANT1 Promoter—The transcriptional regulatory elements of the ANT1 promoter were surveyed using an array of deletion promoter-CAT fusion plasmids (Fig. 1). Plasmid pBLANTCAT1 contains nucleotides at +42 to +123 bp relative to the transcriptional start site at np 0 (11) and incorporates the TATA and CCAAT elements. pBLANTCAT2 extends to −674 bp and includes the OXBOX; pBLANTCAT3 extends to −1.2 kb and encompasses a 22-bp inverted repeat; pBLANTCAT4 extends to −2.0 kb and includes an SV40 core enhancer-like element; pBLANTCAT5 extends to −3.1 kb; and pBLANTCAT6 extends to −8.8 kb. pBLANTCAT5a, pBLANTCAT5b, and pBLANTCAT5c are derivatives of pBLANTCAT5. The 5a fragment was joined at the BglII site. The wild-type promoter was generated with primers 1 and 2.
derivative lacks the +42 to −123-bp TATA and CCAAT box fragments, and 5b and 5c have 1.2 kb of the first intron inserted behind the CAT gene in both orientations.

Transient transfection chloramphenicol acetyltransferase assay of these plasmids revealed three discrete regions, in addition to the CCAAT and TATA elements, which increase transcription 2–3-fold in both myoblasts and myotubes (Fig. 1). The first region is located between −123 and −674 bp (pBLANTCAT1 versus pBLANTCAT2). The second region is located between −2.6 and −3.1 kb (pBLANTCAT2 versus pBLANTCAT5). The third region is located between −3.1 and −8.8 kb (pBLANTCAT5 versus pBLANTCAT6). A negative regulatory region may be located between −674 bp and −1.2 kb (pBLANTCAT2 versus pBLANTCAT3) (Fig. 1).

The TATA and CCAAT elements were shown to be absolutely required for transcription initiation since their removal abolished all CAT expression (pBLANTCAT5a; Fig. 1). We also observed a 2–3-fold increase in CAT expression with all of the promoter constructs except pBLANTCAT1 in myotubes relative to myoblasts (Fig. 1).

Characterization of OBOX Function by PCR Linker Ablation—The OBOX was shown to be the positive regulatory element between −123 and −674 bp by using PCR linker ablation mutants (Fig. 2). PCR was used to prepare a 721-bp fragment without (pBLOB-CAT) and with (pBLOB+CAT) the OBOX, cloned into pBLCAT3. In pBLOB-CAT, the OBOX was substituted with a BgZII site while the length of the DNA and all other sequences were preserved (Fig. 4). pBLOB-CAT and pBLOB+CAT were used to prepare a second set of plasmids, pBLOB-CAT and pBLOB+CAT, by the addition of the region −674 bp to −3.1 kb containing the second positive regulatory element (Fig. 4).

Transfections of the two sets of OBOX-plus and OBOX-minus plasmids into mouse myogenic C2C12 cells revealed marked reduction in CAT activity when the OBOX was ablated (Fig. 3). In multiple experiments, ablation of the OBOX reduced transcription 2–3-fold (Fig. 4). This was true whether or not the second positive element was present. Hence, the OBOX is a positive transcriptional element in muscle cells, and its action is additive relative to the other promoter elements.

Transfection of the two sets of OBOX-plus and OBOX- minus plasmids into epithelially derived HeLa cells yielded very low CAT activity, and the level of expression was the same with or without the OBOX. In three independent experiments using pSV&AT (27) as the positive control (100%), pBLCAT3 gave a mean value of 0.5 ± 0.4% of control and pBLOB-CAT and pBLOB+CAT gave 3.8 ± 2.4 and 3.7 ± 1.9% and pBLOB-CAT and pBLOB+CAT gave 2.4 ± 2.1 and 2.8 ± 2.4%, respectively. These results demonstrate that the AN1 promoter functions poorly in nonmuscle cells and proves that the OBOX sequence is a muscle-specific positive regulatory element.

Fig. 3. ANT1 promoter activity with and without OBOX. A, chloramphenicol acetyltransferase assay. In each lane, the two top spots represent acetylated chloramphenicol; the bottom spot represents nonacetylated chloramphenicol. B, plasmid copy number. Hirt (23) extracted plasmids were digested with EcoRI and hybridized to the pBLCAT3 probe.

OXBOX Binding Factors—To determine if the OBOX interacts with specific nuclear factors, the 40-bp region of the AN1 promoter containing the OBOX was labeled and incubated with nuclear extract prepared from C2C12 cells. Several DNA-protein complexes were observed as bands in a gel mobility shift assay (Fig. 5). One of these complexes was shown to be specific for sequences containing the OBOX (indicated by the arrow). The addition of small quantities of “cold” OBOX probe removed this band (lanes 3–5), whereas a 35-bp nonspecific oligonucleotide which lacked the OBOX sequence had less effect on the complex (lanes 6–8). Hence, mouse C2C12 nuclei appear to contain a nuclear factor which specifically binds to the OBOX probe.
DISCUSSION

Three major positive cis-acting elements were identified in the ANT1 promoter which function in the C2C12 myoblast cells and result in a high level of reporter gene expression. Endogenous ANT1 mRNA was also detected in C2C12 myoblasts by Northern blot analysis.\(^5\) CAT activity was also found to be 2–3-fold higher in C2C12 myotubes than in myoblasts. A similar increase was seen in endogenous ANT1 mRNA levels of C2C12 myoblasts versus myotubes by Northern blot.\(^6\) The myoblast-to-myotube increases may result either from an increased transcription rate or simply a differential accumulation of mRNA.

The three positive elements were functionally additive and independent. The first element was shown to be the OXBOX, located between -452 and -464 bp. Since the OXBOX is found in both the ANT1 and ATPsynβ genes, it joins the recently identified enhancer-like consensus element (5′-TA-GAGACAAGGTTTCACCA) in ATPsynβ, cytochrome c\(_1\), the pyruvate dehydrogenase E1 α subunit gene (28) and the NRF-1 element (5′-GCCGGCATGCGCGGCACC) in cytochrome c (29) as one of a family of transcriptional control elements specific for OXPHOS genes.

Several lines of evidence suggest that the OXBOX is a tissue-specific regulatory element for expression of OXPHOS genes in heart and skeletal muscle. First, the ANT1 OXBOX element functions in C2C12 cells, but not in HeLa cells. Second, the OXBOX sequence has been found in human ANT1 and ATPsynβ genes, and both genes have maximal levels of expression in heart and skeletal muscle (11, 14, 17). Finally, the recently published sequence of the human ANT3 gene, a non-heart-skeletal muscle isoform, does not possess the OXBOX sequence (15). Thus, the OXBOX increases transcription of selected OXPHOS genes in heart and skeletal muscle to be identified.

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