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Identification of the Human Aldolase A Gene as the First Induced Target for the TR2 Orphan Receptor, a Member of the Steroid Hormone Receptor Superfamily

Chawnshang Chang,[1] Han-Jung Lee, and Yi-Fen Lee

University of Wisconsin Comprehensive Cancer Center, Madison, Wisconsin 53792; and Department of Pathology, Department of Urology, and Department of Biochemistry and Cancer Center, University of Rochester, Rochester, New York 14624

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The human TR2 orphan receptor (TR2) is a member of the steroid/thyroid hormone receptor superfamily that regulates the transcription of complex gene networks and subsequently controls diverse aspects of growth, development, and differentiation. In the present study, we have found that the TR2 is one of the M1 site (nucleotide numbers 2017-2034, 5′-AAAAGGGCAGGGGTCATT-3′) binding proteins of the muscle-specific pM promoter in the human aldolase A gene. Electrophoretic mobility shift assay (EMSA) showed a specific binding with high affinity (dissociation constant = 4.6 nM) between the TR2 and the M1 element. Circular permutation assay revealed a localized DNA flexibility induced by the TR2 binding, and the bend angle was estimated to be 73 ± 2°. Furthermore, a dual-luciferase reporter gene assay demonstrated that the TR2 may enhance the expression of luciferase activities via the wild-type M1 site but not the mutant M1 element in human QM7 muscle myoblasts. In conclusion, our data represent the first case of demonstrating that the TR2 may serve as a transcriptional inducer in muscle-specific aldolase A gene expression.

The human TR2 is one of the first orphan receptors identified that shares structural homology with members of the steroid/thyroid hormone receptor superfamily (1, 2). The TR2-11 cDNA encodes a protein of 603 amino acid residues with a calculated molecular mass of 67 kilodaltons (kDa). The deduced amino acid sequences of the DNA-binding domain of the TR2 show 50-60% homology with other known steroid receptor members (2-4). However, the amino acid sequences of the putative ligand-binding domain of the TR2 show low homology (10%) with known receptors. In addition, the TR2 mRNA has been detected in many rat tissues with the highest abundance in male reproductive organs, such as ventral prostate, seminal vesical, and testis (1). Interestingly, the expression of the TR2 mRNA is negatively regulated by androgen in the human prostate LNCaP cell line and rat ventral prostate (1, 5).

Differential regulation of target genes by members of the steroid hormone receptor superfamily is determined by at least three properties: protein-DNA interactions, protein-protein interactions, and the protein environment (6). First, protein-DNA interactions are mediated by the highly conserved DNA-binding domain that defines the steroid receptor superfamily. The molecular specificity of the steroid/thyroid hormone receptors is achieved through their selective interaction with hormone response elements (HREs), which are structurally related but functionally distinct. The TR2, containing the EGCKG amino acid sequence in the proximal (P) box of the DNA-binding domain, has been grouped into members of the estrogen receptor subfamily, recognizing the hexameric AGGTCA consensus motif (7). Consequently, we have demonstrated that the TR2, serving as a transcriptional repressor, may recognize two direct repeats of the AGGTCA core consensus motif in several target genes (8, 9, 10).

Second, protein-protein interactions necessary for the formation of homo- and/or heterodimers in solution are mediated by at least two regions. The first region is a DNA-supported asymmetric dimerization interface located within the DNA-binding domain of the receptors that selectively promote DNA binding to cognate direct repeat HREs (11-13). The second one is the identity (I) box in an extensive C-terminal dimerization interface in the ligand binding domain (14). The combi-

[1] Correspondence should be addressed to Dr. Chawnshang Chang, Department of Pathology, University of Rochester, 601 Elmwood Ave., Box 626, Rochester, NY 14642. Fax: (716)2731027.

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nation of this obligatory I box and an optional dimerization interface of the DNA-binding domain increases the diversity of the heterodimeric interaction and high-affinity DNA binding.

Third, the protein environment indicates that, aside from the receptor and the DNA themselves, additional multiple parameters also control the selectivity of steroid/thyroid hormone receptors for recognition of their target genes (6). For example, ligand specificity is one of the key parameters contributing to DNA-binding and dimerization. Studies of thyroid receptor (T_3R) and RXRs suggest that their respective ligands may affect the formation of dimerization and diversity of function (15, 16). Furthermore, nuclear accessory factors are thought to serve as bridging molecules or adaptors between the steroid hormone receptors and the basal transcriptional machinery (17, 18).

Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) isoenzymes (type A in muscle, type B in liver, and type C in brain) are a family with tissue-specific control of gene expression (19). The human aldolase A gene is transcribed from at least three different promoters, pN, pM, and pH, generating mRNAs with a common coding sequence but different 5′ flanking regions (19-24). In transgenic mice, both pN and pH promoters are active in many tissues, while the pM promoter is restricted to adult muscles composed mainly of fast-twitch fibers (25). Recently, a minimal pM promoter has been defined that consists of two important DNA elements (26). The first binding site (MEF2/NF1) is for myocyte enhancer factor-2 (MEF2) members and NF1 factors. However, the second one (M1) is for unknown proteins. Surprisingly, this M1 site contains two direct-repeats of the AGGTCA consensus motif spacing with one base-pair (bp), referred to as a DR1 sequence. Based on our previous data, we strongly suspected that the TR2 could be one of the unknown proteins which may bind to this M1 site and influence the tissue-specific gene expression of the pM promoter. Hence, we explored the specific interaction between the M1 site and the TR2. Moreover, the possible consequences of tissue-specific expression via protein-DNA binding in the human QM7 muscle myoblasts were analyzed.

MATERIALS AND METHODS

Plasmid construction. Plasmid pSG5-TR2 contains the full-length coding sequence of the human TR2 cDNA in the pSG5 vector (Stratagene) under the control of both SV40 and T7 promoters for *in vivo* and *in vitro* expression, respectively (8, 10). Plasmids pCMX-RXRα and pT7hmSHP were kindly provided by Drs. Ronald M. Evans and Wongi Seol, respectively (27, 28). Plasmids pGL3-M1 and pGL3-mM1 consist of one copy of the wild-type M1 site corresponding to nucleotide numbers 2017-2034 (5′-AAAAGGGCAGGGGTCATT-3′) and mutant M1 site (5′-AAAAGCGCAGGGCTCATT-3′) of the pM promoter of the human aldolase A gene, respectively (20).

Coupled in vitro transcription/translation and EMSA. Plasmids containing the full-length TR2, RXRα and SHP cDNAs were *in vitro* transcribed and translated directly by the TNT system (Promega) for EMSA as previously described (8).

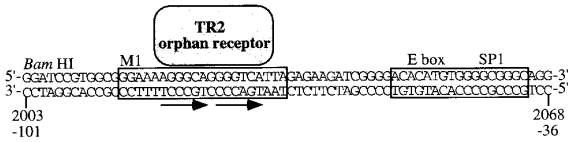

FIG. 1. Schematic structure of the M1 site of the pM promoter in the human aldolase A gene bound by the TR2. The putative protein binding sites are boxed, and the arrows display the DR1 sequence. Numbers under the sequence show the aldolase A gene nucleotide residues and the relative 5′ flanking sequence from the transcriptional initiation site of the pM promoter, respectively (26).

Circular permutation assay. Circular permutation assay was carried out as previously described (29). Generally, circularly permutated DNA fragments were isolated by restriction enzyme digestion of pBend-M1, and purified by electrophoresis on a 1% agarose gel. DNA fragments were radiolabeled by α-[^{32}P]dCTP (Dupont NEN) with a random primed DNA labeling kit according to the manufacturer's instructions (Boehringer Mannheim). EMSA was then conducted essentially as described above. For the determination of bend angles, the angle of curvature induced in the DNA by protein binding was calculated using the empirical equation as previously described (30). Thus, the equation is $\mu M/\mu E = \cos(\alpha/2)$, where α displays the bending angle induced by the protein binding. μM and μE correspond to the electrophoretic mobility for the protein-DNA complexes when the binding site is at the middle and at the end of the DNA fragment, respectively.

Cell culture and transfection experiments. The human QM7 muscle myoblast cells (American Type Culture Collection, CRL-1962) were cultured in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), amphotericin (0.25 μg/ml), and 15% heat-inactivated (56 °C for 30 min) fetal bovine serum (Harlan). Transient transfection of the QM7 cells plated at an initial density of 3×10^5/60-mm dish was carried out by the calcium phosphate precipitation method in the presence of 15% fetal bovine serum or charcoal-treated serum (CTS) as described previously (8, 31-33). Dual-luciferase reporter assay system was conducted according to the manufacturer's instructions (Promega). The pRL-TK plasmid (Promega) was co-transfected to normalize the transfection efficiency.

RESULTS

Binding of the human TR2 to the M1 site of the pM promoter of the aldolase A gene. To test if the TR2 could be one of the unknown proteins regulating the tissue-specific gene expression of the aldolase gene, we applied EMSA to reveal the specific interaction between the TR2 and the M1 site. As illustrated in Fig. 1, the TR2 and several orphan receptors were expressed in a coupled *in vitro* transcription-translation system (Fig. 2A). The TR2 was expressed as a protein with a molecular mass of 67 kDa (lane 1), while the mock-translated control produced no detectable product (lane 2). In order to investigate the possible protein-protein interaction between the TR2 and other orphan receptors in the recognition of the M1 site, RXRα and SHP orphan receptors were also generated *in vitro* for

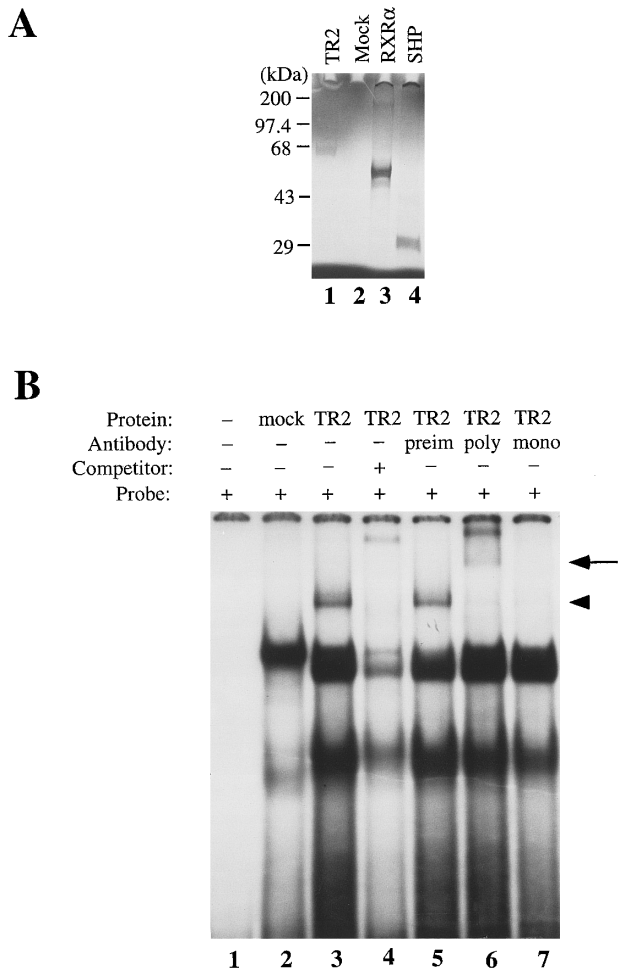

FIG. 2. Analysis of *in vitro* expression of the TR2 and binding of the TR2 to the M1 site of the pM promoter in the human aldolase A gene. (A) Analysis of *in vitro* translated proteins by SDS-10% polyacrylamide gel electrophoresis. The TR2 (67 kDa), mock-translated product, RXRα (51 kDa), and SHP (29 kDa) expressed in a coupled transcription-translation system are displayed in *lanes 1-4*, respectively. (B) Binding of the TR2 to the M1 site of the aldolase A gene. EMSA was performed with the *in vitro* expressed TR2 and the ^{32}P-labeled DNA probe. Lane 1 displays the probe alone, which contains the 18-bp M1 site sequence (26). Binding reaction mixtures incubated with the probe and either mock-translated product (lane 2) or the *in vitro* synthesized TR2 (lanes 3-7) in the presence of 100-fold molar excesses of unlabeled oligonucleotides (lane 4), preimmune serum (preim, lane 5), polyclonal anti-TR2 antibody (poly, lane 6), or monoclonal anti-TR2 antibody (mono, lane 7) are shown. The retarded complexes are indicated by the arrowhead for specific DNA-protein complexes, whereas the supershift band is marked by the arrow for DNA-protein-antibody complexes. Nonspecific complexes appear between the retarded complexes and the free probe at the bottom.

later study (lanes 3 and 4). Using EMSA, the potential interaction of the TR2 with the M1 site of the aldolase A gene was revealed as shown in Fig. 2*B*. Double-stranded oligonucleotides corresponding to nucleotide numbers 2017-2034 of the aldolase A gene were labeled as a probe. A specific DNA-protein complex was visualized in EMSA (Fig. 2B, lane 3, arrowhead), and could be abolished in the presence of 100-fold molar excesses of unlabeled oligonucleotides (lane 4). In addition, the polyclonal anti-TR2 antibody could supershift this specific DNA-protein complex (lane 6, arrow). However, the monoclonal anti-TR2 antibody might eliminate this complex (lane 7). These data demonstrated that the TR2 can specifically bind and form a single complex with the M1 site of the aldolase A gene.

To further determine the DNA-protein binding affinity between the TR2 and the M1 site, Scatchard binding analysis was conducted by the EMSA as shown in Fig. 3. Constant amounts of the *in vitro* expressed TR2 (5 μl) were incubated with different concentrations of the DNA probe (0.2-6.4 ng). Specific DNA-protein complexes were resolved in EMSA (Fig. 3A, arrowhead). Scatchard analysis resulted in a single binding component for the DNA-protein complex with a dissociation constant (K_d) of 4.6 nM and B_{max} of 0.03 nM (Fig. 3B). These data fit well into the range of K_d for classical steroid receptors with their HREs, suggesting that the TR2 can bind to the M1 site with high affinity.

Lack of indication that the TR2 forms heterodimers with other partners. To reveal whether the TR2 might form heterodimers with either RXRα or SHP by protein-protein interaction, we employed *in vitro* expression (Fig. 2A) and EMSA in the presence of the M1 element (Fig. 4). A specific DNA-protein complex was seen, as before, between the TR2 and the probe (Fig. 4, arrowhead). In contrast, both RXR and SHP do not interact with the M1 site (lanes 4 and 5). Moreover, we detected the original DNA-protein complexes only when the DNA probe was incubated with the TR2 and either RXR or SHP (lanes 6 and 7). Subsequently, the polyclonal anti-TR2 antibody could further supershift the DNA-protein complex formed by the TR2 and the probe (lane 8). These results suggested that heterodimerization does not occur between the TR2 and either RXR or SHP.

DNA bending feature. DNA bending has been observed for several cases in transcriptional regulatory members (29, 30). The TR2 showed high affinity and sequence-specific DNA binding to the M1 site of the aldolase A gene. A circular permutation assay was applied to see if the TR2 may affect the localized conformation of the M1 element due to protein-DNA binding. Annealed 18-bp oligonucleotides containing the M1 site of the aldolase A gene were positioned near the center of two tandemly repeated copies of a polylinker sequence in the pBend5 plasmid. Digestion of the resulting plasmid, pBend-M1, with a number of restriction enzymes yielded a set of DNA fragments with almost identical length but containing the M1 site at different positions (Fig. 5A). Each DNA fragment was incubated with either mock-translated product or the

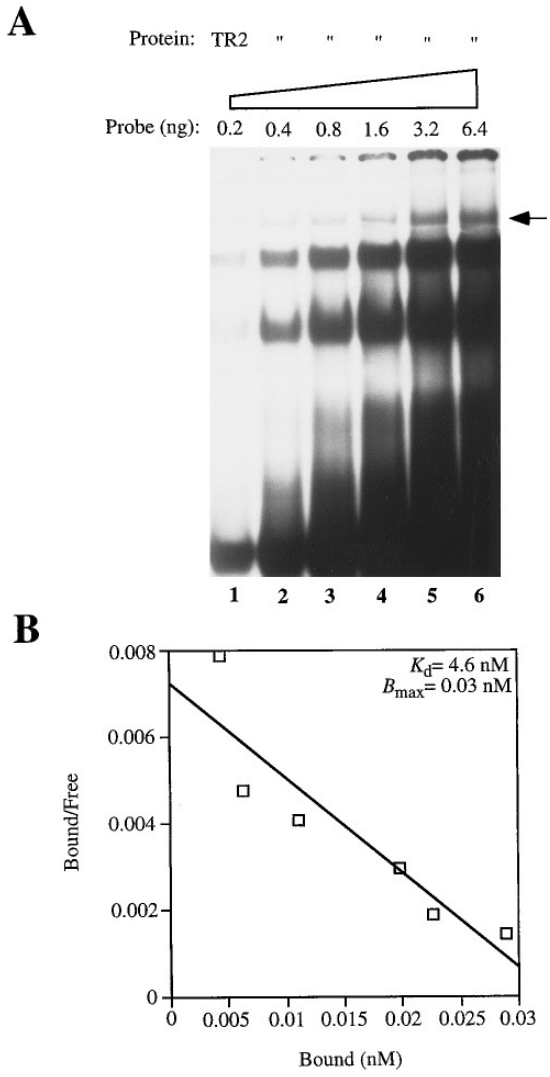

FIG. 3. Binding affinity of the TR2 to the M1 site of the aldolase A gene. (A) Binding of the TR2 to different concentrations of the probe in EMSA. Constant amounts of the *in vitro* expressed TR2 (5 μl) were incubated with various amounts of the probe (0.2-6.4 ng). The specific protein-DNA complex indicated by the arrow and the free probe at the bottom were quantified by PhosphorImager (Molecular Dynamics). (B) Scatchard plot analysis. The ratio between specific DNA-protein binding (bound, nM) and free DNA probe with respect to specific DNA-protein binding (bound/free) was plotted. The dissociation constant (K_d) and B_{max} values were generated from Ebda program (Biosoft).

TR2 to form complexes resolved in a 5% nondenaturing gel (Fig. 5B). Figure 5*C* shows that the relative mobility was plotted against the position of the recognition sequence within the DNA fragment. The result revealed a DNA bending can be induced by the binding of the TR2, and the bend angle was estimated to be 73 ± 2°.

Induction of the aldolase A gene expression by the TR2. To examine whether the TR2 plays a regulatory role in the aldolase A gene expression via the interaction with the M1 site, we performed a dual-luciferase reporter assay in human QM7 muscle myoblast cells. As shown in Fig. 6, QM7 myoblasts were co-transfected with increasing amounts of mammalian expression vector containing full-length TR2 cDNA (pSG5-TR2) and one of three reporter plasmids (the parent pGL3-promoter, pGL3-M1, and pGL3-mM1). The results showed that the TR2 can trigger the luciferase reporter activity only via the wild-type M1 element in a dose-dependent manner. This indicated that the TR2 can enhance aldolase A gene expression via the interaction between the TR2 and the M1 site of the pM promoter.

DISCUSSION

The human aldolase A gene identified in the present study may represent the first induced target for the TR2. We have previously demonstrated that the TR2 may repress the gene expression of several targets, including the human erythropoietin gene, SV40 major late promoter, and RAR/RXR signal pathways, respectively (8, 9, 10). However, the data in this report demonstrated that the TR2 can also function as an inducer to induce its target gene. The ability and behavior of the TR2 are, therefore, in good concert with other orphan receptors, like the COUP-TFs and RXRs (4, 6). Thus, various superfamily members recognize and function differentially in complex networks of gene-specific regulation.

The TR2 may be one of candidates to bind to the M1 site of the pM promoter of the aldolase A gene and regulate the tissue-specific gene expression. According to data from previous studies, the minimal pM promoter contains at least two important DNA elements

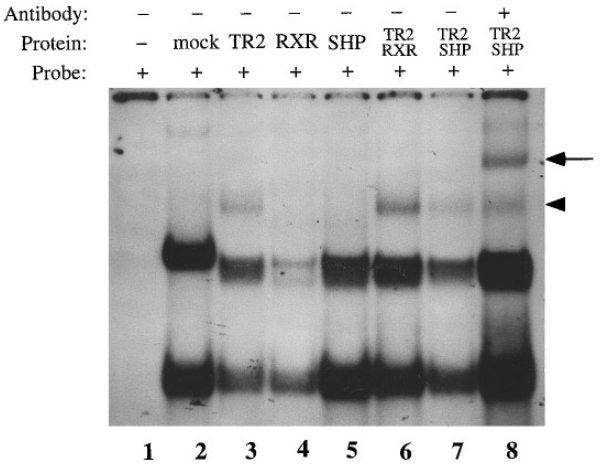

FIG. 4. Binding of the TR2 with other partners to the M1 site of the human aldolase A gene. Lane 1 displays the probe alone. Binding reaction mixtures incubated with mock-translated product (lane 2), the TR2 (lane 3), RXR (lanes 4 and 6), or SHP (lanes 5, 7, and 8) in the presence of polyclonal anti-TR2 antibody (lane 8) are shown.

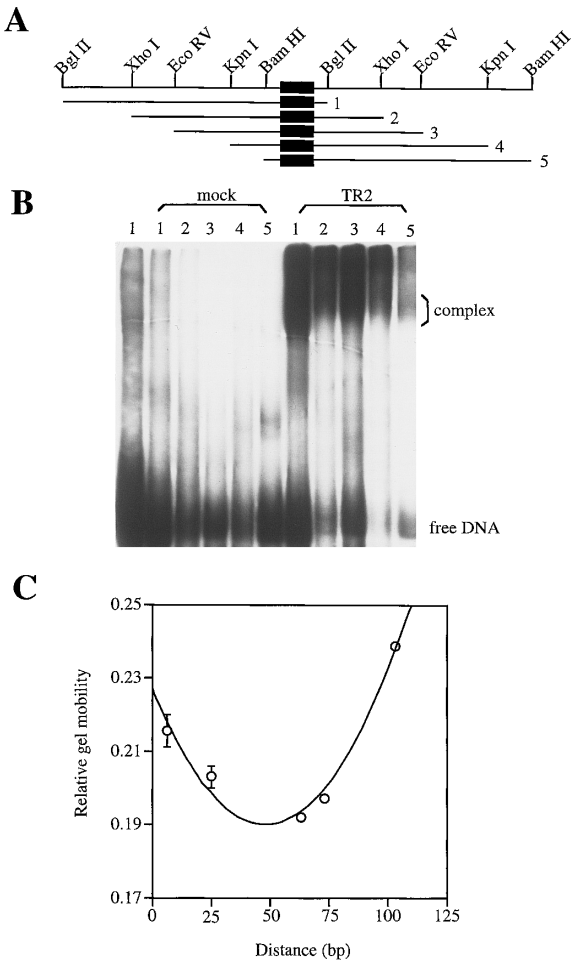

FIG. 5. Circular permutation assay of the DNA bending induced by the binding of the TR2 to the M1 site of the aldolase A gene. (A) Probes used in circular permutation assay. Plasmid pBend-M1 containing 18-bp of the M1 site (filled box) flanked by tandemly duplicated DNA sequences was cleaved at the restriction enzyme sites indicated. The isolated and purified DNA fragments (designated 1-5) represent DNA products from *Bgl* II, *Xho* I, *Eco* RV, *Kpn* I, and *Bam* HI, respectively. (B) EMSA using each of the circularly permutated DNA fragments 1-5 bound by either mock-translated product or the TR2. A control reaction in which no protein was added to fragment 1 is also shown. (C) Analysis of the induced DNA bends. Relative gel mobility (migration of protein-DNA complex/migration of free DNA) was plotted against distance of the M1 site from the 5′ end of the DNA fragment.

(25, 26). The MEF2/NF1 site is essential but not sufficient for the enhancer activity in skeletal muscle-specific gene expression (34). This implies that the M1 site is indeed needed in order to fulfill the muscle-specific enhancer and promoter activities. However, we do believe that the TR2 may represent one of the M1 binding proteins. It will not be a surprise, if other receptors or proteins are also capable of binding to this M1 site, and differentially regulating on this region. Especially, orphan receptors, such as COUP-TFs, HNF4, TR4, and RXRs, may play a role via the M1 site of the aldolase A gene. These orphan receptors are able to bind to DR1 sequences and regulate their targets (6, 27). In addition, it is very likely that several M1 binding proteins can integrate and coordinate with this element of the pM promoter to regulate the aldolase A gene in physiological conditions. These M1 binding proteins may act alone or interact with one another to yield additional or synergistic effect.

Basically, members of the steroid/thyroid hormone receptor superfamily are capable of binding to their DNA response elements in three different ways, homodimeric, heterodimeric, and monodimeric categories (4, 6). The NGFI-B/TR3 orphan receptor is a classical example for the monomeric subfamily members that bind to a single copy of the core motif proceeded by an AT-rich sequence, referred to as a NGFI-B response element (NBRE) (35-37). The M1 site of the pM promoter in the aldolase A gene contains a DR1 sequence as we indicated in Fig. 1. Indeed, the first repeat of the core motif corresponding to nucleotide numbers 2017-2025 consists of a NBRE-like sequence. We suspected that this NBRE-like motif may contribute to the recognition for the TR2 during tissue-specific regulation. Therefore, the NBRE-like oligonucleotides were synthesized and tested for the recognition by the TR2 using EMSA

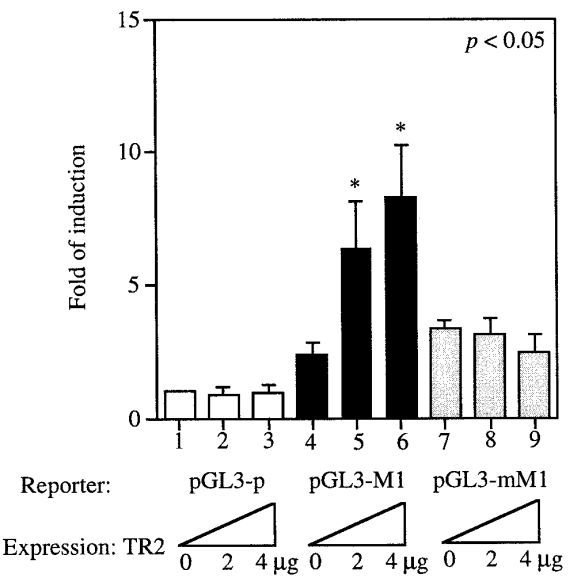

FIG. 6. The TR2 activates the aldolase A gene expression via the M1 site by dual-luciferase reporter assay. Human QM7 muscle myoblast cells were co-transfected with increasing amounts of the pSG5-TR2 expression plasmid (lanes 2, 3, 5, 6, 8, and 9) and the parent pGL3-promoter (lanes 1-3), pGL3-M1 (lanes 4-6), or pGL3-mM1 (lanes 7-9) reporter plasmid. All firefly luciferase activities were normalized with *Renilla* luciferase activities and then averaged over at least four independent experiments with error bars designating standard deviations. Significant ($p < 0.05$) differences from control (lane 1) are marked with asterisks.

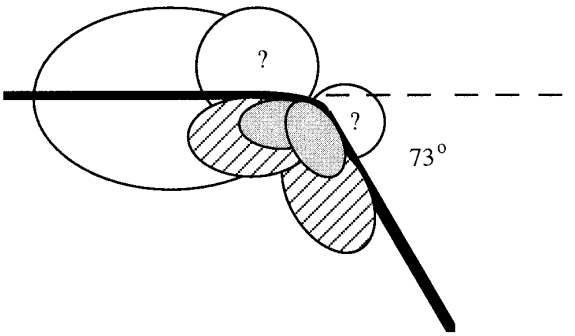

FIG. 7. Model for the TR2-induced DNA bending. The TR2 is mainly composed of the N-terminus, DNA-binding domain (dashed), and C-terminal region (shaded). Other receptor-associated proteins (question marks) and/or transcriptional machinery (blank circle) may associate with the TR2 around the M1 DNA template (broad line).

(data not shown). The results concluded that the TR2 is not able to bind to the NBRE-like element. This ruled out the possibility that the NBRE-like motif alone may be involved in the regulation of the aldolase gene by the TR2.

Complex protein-DNA binding are responsible for the storage and expression of genetic information (29). DNA bending in response to regulatory protein binding is a largely unexplored phenomenon in the mechanism of gene control. Steroid hormone receptor members which normally bind as homodimers or heterodimers have previously been shown to bend DNA (38). The bend angles induced by these receptors appear to be: estrogen receptor, ~50°; T_3R-RXR, ~65 to 75°; RXR-RXR, ~92°; retinoic acid receptor-RXR, ~57 to 63°; and RORα, ~130° (38). We have demonstrated that the TR2 is a transcriptional activator for muscle-specific aldolase A gene expression. In addition, the specific binding of the TR2 toward the M1 site is involved in localized DNA flexibility (Fig. 7). Although the molecular mechanism of stimulation in such a gene regulatory system is still not known, two general models have been hypothesized. The first suggests that direct interaction between the TR2 and transcriptional machinery results in stronger binding of RNA polymerase to the promoter. For the second one, a DNA structural transition is needed. This may be involved in the alternation of the double helix DNA structure or stability in the adjacent RNA polymerase binding site. Although no function for DNA bending by the steroid hormone receptors has been directly demonstrated, it is likely that protein-protein interaction with other accessory factors or the basal transcriptional machinery is also required for transcriptional activation.

To understand the potential physiological properties and possible role in cellular responses and embryonic development of the human TR2 is our long-term goal. In the search for target genes of the TR2, we may be able to identify new response systems that may have valuable biological and physiological implications. Thereby, we may reveal the association of this orphan receptor in different physiological pathways, which may ultimately lead to the discovery of its ligand specificity and physiological functions.

ACKNOWLEDGMENTS

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