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Identification of an Essential *cis*-acting Element (TR2-PACE) in the 5' Promoter of Human TR2 Orphan Receptor Gene

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The human TR2 orphan receptor (TR2) is a member of the steroid/thyroid hormone receptor superfamily. It has been shown to be expressed in a wide variety of tissues during development. Using deletion mutation analyses and transient transfection CAT assays, we demonstrated here that a DNA fragment of 103 bp, with a sequence from +65 to –38, containing an initiator is capable of serving as a core promoter to initiate basal level transcription; further extending of this core promoter sequence up to –441 maximizes the reporter gene expression. Within this positive regulatory region (–441/+65), we were able to narrow the regulation-responsible sequence down to a small 64-bp (–263/–201) DNA fragment named the TR2 promoter activating *cis*-element (TR2-PACE). Further deletion mutagenesis and shifting of the insert position followed by reporter assays demonstrated that this TR2-PACE is essential for high-level induction of a heterologous core promoter's activity in a position-dependent nature. In addition, orientation tests indicated that the sense, but not antisense orientation increased the TR2 core promoter activity. Moreover, electrophoresis mobility shift assays and Southwestern analyses suggested that TR2-PACE may interact with unknown specific nuclear proteins for its enhancer activity. Together, our data suggest that TR2-PACE is a position-dependent and, in the case of TR2 core promoter (TATA-less), an orientation-dependent *cis*-activating element required for maximal expression of the TR2 gene.

Key Words: Nuclear receptor; steriod receptor; TRH; TR2-11.

Introduction

Steroid hormones function in precise biological programs via their specific receptors, which are ligand-activated transcription factors *(1,2)*. These proteins thus link extracellular cues for reproduction, development, and homeostasis directly to gene regulation. In addition to those receptors involved in mediating specific ligand transduction systems, a large proportion of this gene superfamily consists of putative receptors known as orphan receptors, which may bind to unidentified ligand(s)/hormone(s). A highly conserved DNA-binding domain (DBD) and a carboxy-terminal ligand-binding domain (LBD) characterize members of this family. Most of these orphan receptors have been discovered by cross-hybridization with authentic steroid receptor cDNAs *(1,3)*. The cDNA of the human testicular receptor 2 (TR2) was isolated in the screening of human testis and prostate cDNA libraries using an oligonucleotide probe homologous to the DBD of the steroid hormone receptor (SR) *(3)*. Three TR2 subclasses, TR2-5, TR2-7, and TR2-9, were isolated from a human testis library, and the fourth clone, TR2-11, was identified in a human prostate library. These receptors are found to share identical sequences in both the N-terminal domain and putative DBD, suggesting that all TR2 subtypes may act on the same hormone response elements (HREs), but differ in the length of their LBD *(4)*. Chromosome mapping indicated that the TR2 gene is mapped to human chromosome 12 at band q22 *(5)*. Additionally, the genomic intron-exon junction information, and the identification of specific junction sequences for splicing sites, clearly suggested that the existence of multiple isoforms of the TR2s were derived from alternative splicing *(5)*. The major cap site which is surrounded by an initiator (Inr) sequence is located at the 104th nucleotide upstream to the translation start codon *(5)*.

Among all adult mouse tissues, the testis is the organ that expresses the highest level of TR2 proteins. Similarly, in rat tissues the TR2 is expressed with higher abundance in male reproductive organs *(3)*. However, *in situ* data on the TR2 gene expression pattern during mouse embryogenesis has also revealed that there is a strong and wide

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expression of the TR2 gene throughout the development of the animal *(6)*. Note that there also is an intense expression of TR2 among the developing neural system *(6)*. Collectively, these observations suggest that the TR2 gene may play an important role in diverse aspects of development including the neural system formation, organogenesis, cell proliferation and differentiation.

The transcription of TR2 mRNA is negatively regulated by androgen in the human prostate LNCaP cell line and in rat ventral prostate *(3,7)*. Recently, our data demonstrated that irradiation can repress the TR2 gene at both the translational and transcriptional levels in the human MCF-7 cell line. Transient transfection assays further link the tumor suppressor p53 to this repression by proving that endogenously induced or exogenously transfected p53 can repress TR2 gene expression *(8)*. Other studies further suggested that TR2 may represent a master regulator to modulate other signal pathways, such as retinoids/RAR-RXR *(9,10)*, ciliary neurotropic factor *(6,11)*, and thyroid hormone *(12)*. The expression of erythropoietin *(13)*, aldolase A gene *(14)*, and SV40 *(15)* were also regulated by TR2.

Our previous study on the 2.7-kb 5'-flanking region of the TR2 gene revealed that there is an Inr sequence (5'-TTCAACCTCT-3') located upstream to the cap site of the otherwise TATA-less TR2 gene 5'-flanking region *(5)*. Here, we further demonstrate that the –38/+65 region can function as a core promoter for initiating the transcription. Upstream to the core promoter, there is an important control region, spanning –73 to –441, which when included, increased the transcription of the TR2 gene to a much higher level. This *cis*-acting regulatory region of the TR2 gene can be narrowed down to 64 bp (–263/–201), which we called TR2 promoter activating *cis*-element (TR2-PACE). The reporter chloramphenicol acetyltransferase (CAT) activity can be enhanced significantly as long as this DNA fragment is upstream to a core promoter, containing either a TATA box or an Inr, at a sense orientation, suggesting that the TR2-PACE may function in an orientation- and position-dependent fashion. Gel retardation data showed that certain poorly defined nuclear protein(s) can specifically bind to the TR2-PACE DNA and form a high molecular weight complex. Furthermore, Southwestern assay revealed that the TR2-PACE may interact with nuclear proteins isolated from HeLa (~100 kDa in size) and two nuclear proteins from LNCaP (~100and ~35 kDa in size) cells.

Results

Identification of Essential Sequences for TR2 Gene Expression

In an attempt to map the functional core promoter and to identify the essential *cis*-acting elements within the TR2 5'-flanking region, we created a series of progressive deletion mutants of the TR2 gene promoter CAT plasmids (p-2180TR2CAT, p-1857TR2CAT, p-1714TR2CAT, p-1390TR2CAT, p-1354TR2CAT, p-959TR2CAT, p-441TR2-CAT, p-242TR2CAT, p-73TR2CAT and p-38TR2CAT). These plasmids were then transiently transfected into human prostate carcinoma PC3 and breast carcinoma MCF-7 cells to analyze CAT activity. Figure 1A illustrates the relative reporter expression level of the progressive deletion mutants. Whereas the parental vector plasmid, the promoterless pBSCAT2, showed no detectable expression activity, p-2666TR2CAT, the reporter containing the 2.7-kb TR2 5'-flanking sequences, showed strong promoter activity in all cells we tested (PC3, MCF-7, Fig.1A; HepG2, LNCaP, HeLa, Chinese hamster ovary; data not shown). Progressive deletion from –2666 to –441 did not significantly influence CAT activities.

Deletion from bp -441 to -242 reduced promoter activity by approx 70% as compared to the p-2666TR2CAT construct. Further deletion from bp –242 to –73 showed a loss of another 90–95% CAT activity (Fig. 1B, p-242TR2CAT vs p-73TR2CAT). It has been well-demonstrated that an Inr is able to serve as a minimal promoter, and mutations near the Inr can result in reduction of promoter activity. Because there is an Inr sequence (–1/+9) surrounding the cap site of the otherwise TATA-less TR2 gene 5'-flanking region *(5)*, we wonder whether the Inr sequence within TR2 gene is capable of behaving similarly. Therefore, we further deleted from the p-38TR2CAT to construct p+6TR2CAT, the shortest deletion mutant that truncated the Inr sequences, and assayed for the loss of CAT activity. As we expected, the CAT activity dropped to a level comparable to that of the background activity of the parental vector, pBSCAT2 (Fig.1B) indicating that this specific Inr is capable of functioning as a core promoter.

Characterization of the Proximal Enhancer Element

The use of the aforementioned progressive deletion mutants assay revealed a positive regulatory region of 369 bp from –73 to –441 nucleotides within the 2.7-kb 5'-flanking region. To characterize this region further, we generated internal deletion mutants. We then tested these mutants transiently in PC3 cells (Fig.2). We found that the CAT reporter activity was reduced to a level similar to that of the basal level of p-38TR2CAT when a small fragment of 64 bp, spanning from –263 to –201, was internally removed (Δ358, Δ226, Δ135, and Δ64 shown in Fig.2). Conversely, the CAT activities of deletion mutants immediately upstream or downstream to the 64-bp region (Δ71 and Δ91 shown in Fig.2) remained at a peak level comparable to the activity obtained from the full length construct, p-2666TR2CAT. This evidence strongly suggests that the *cis*-acting control element which promotes maximal TR2 gene expression to a level 10-fold higher than the basal level, can be narrowed down to 64 bp, the –263/–201 region.

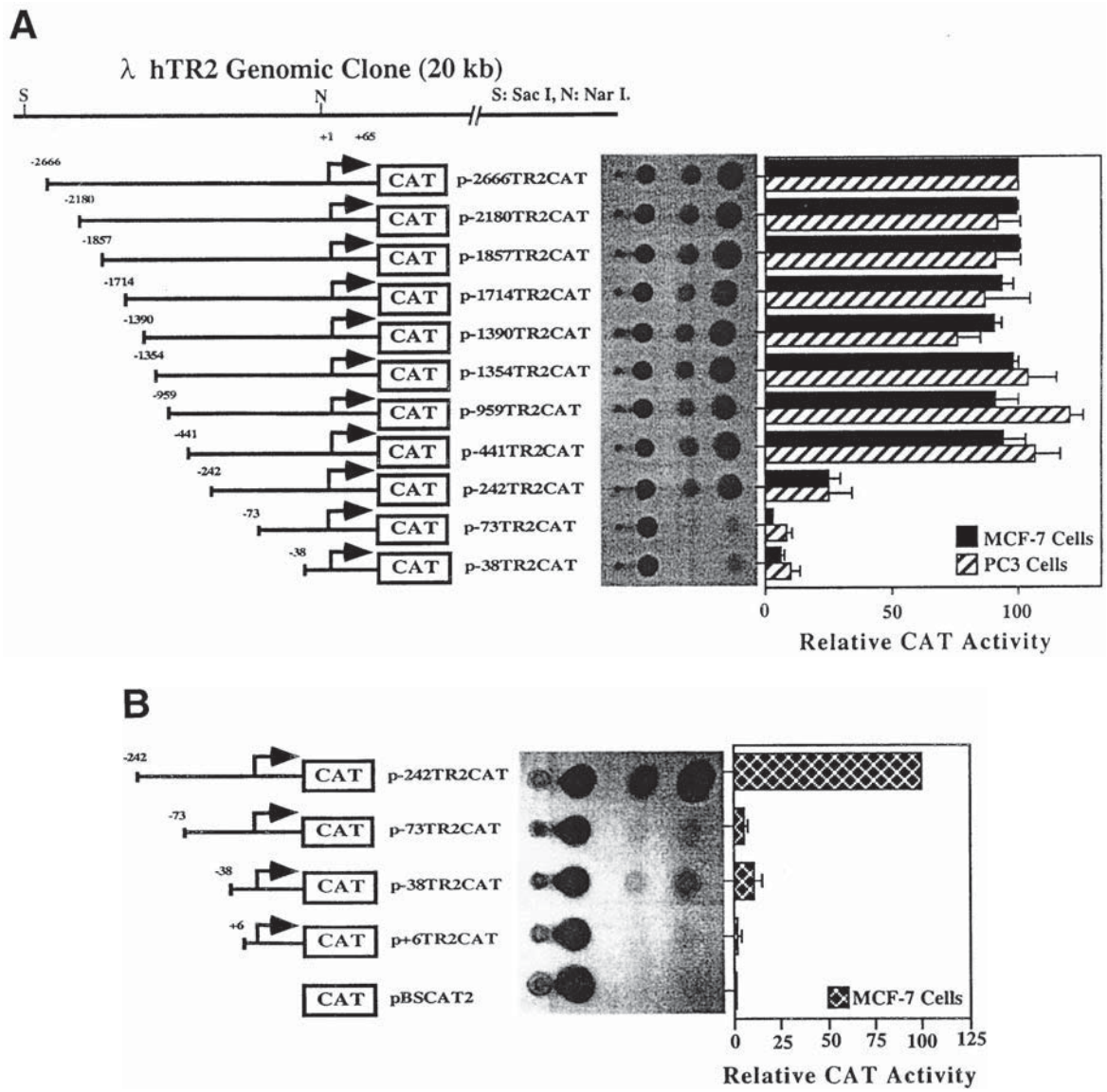

Fig.1. Analysis of the promoter activity. **(A)** The promoter activity of serial deletion mutants of the human TR2 gene. On the left are the deletion constructs, numbered and named according to their length upstream of the cap site. The CAT activity (far right, bar graph), after constructs (3 μg) were transiently transfected (total DNA amount, 10 μg) into cells indicated, was measured and normalized with β-galactosidase activity. The parental vector (pBSCAT2) has no detectable activity. The relative CAT activities of all deletion plasmids were calculated with that of p-2666TR2CAT as 100%. The cell lines tested include MCF-7 and PC3 cells. Data points were the mean value of five separate experiments. The blot (center) is a typical set of CAT assay data obtained from the MCF-7 cells. **(B)** The promoter activity of the proximal serial deletion mutants of the human TR2 gene. The relative CAT assay results of the four shortest deletion mutants were shown to compare with the background CAT activity of the pBSCAT2. The reporter activity of the p-242TR2CAT was plotted as 100%. These results represent the mean value ± SE of five experiments.

Major cis-Acting Element Potentiates Simian Virus 40 Minimal Promoter in Position-Dependent Manner

An interesting property of many transcription factor binding sites is that they can function as regulated enhancer elements when arranged as tandem repeats of multiple copies and placed upstream from a heterologous promoter (the viral minimal promoter is usually used for this purpose). In addition, the relative positions and orientations of the enhancer are not usually critical for the enhancer function. To examine whether this major *cis*-acting region possesses a similar characteristic, we inserted separately, three fragments which share the overlapping region of the 64-bp in a sense orientation (Fig.3A), into a reporter construct in which the CAT gene is driven by the simian virus 40 (SV40) minimal promoter containing a TATA box (pCATp; Promega). The data shown in Fig.3B exhibit the

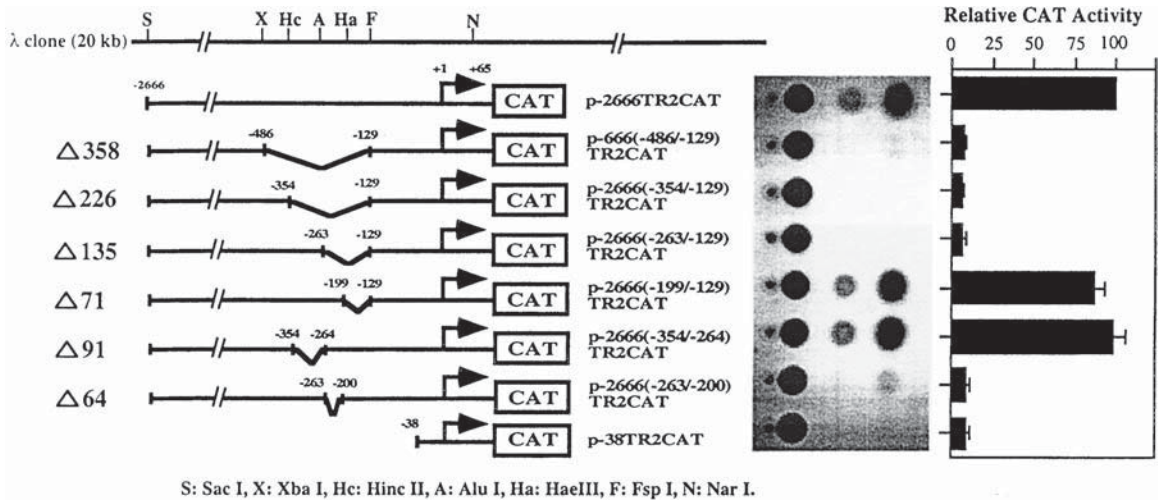

Fig.2. Internal deletion analysis of the enhancer activity of the proximal enhancer region. On the left are the deletion constructs that have sequences removed internally from p-2666TR2CAT, numbered and named according to the length and the position of the region deleted upstream of the cap site. The CAT activity, (far right, bar graph) after constructs (3 μg) were transiently transfected into PC-3 cells, was measured and normalized with β-galactosidase activity. The relative CAT activities of all deletion plasmids were calculated with p-2666TR2CAT as 100%. Data points were the mean value ± SE of five separate experiments. The blot (center) is a typical set of CAT assay data. Restriction enzyme sites that were utilized to construct these deletion mutants are depicted at the top for the relative position and at the bottom for the names of the enzymes.

enhancing ability of these constructs when tested in PC3 cells. In comparison to the CAT activity shown by the control plasmid pCATp (plotted as one fold), p64(+)(–262/–201)CATp induced the activity to sevenfold (Fig.3B. and Fig.3C, lanes 1 and 2, respectively). The induction by p133(+)(–262/–130)CATp was 8 fold, whereas 13- to 14-fold CAT activity was observed with p362(+)(–491/–130)CATp (Fig.3B, lanes 3 and 4, respectively). Therefore, these results demonstrate that the 64-bp region is capable of behaving as an enhancer with respect to the SV40 promoter activity. The sequences near the 64-bp region can contribute to increase the strength of the enhancer, presumably by helping to stabilize the DNA *trans*-acting factor complex association.

We then tested whether the ability of the 64-bp region to induce transcription is dependent on its orientation and position in pCATp. Placing the 64-bp fragment upstream of the SV40 promoter resulted in increased CAT activity (Fig.3C, lanes 2–4) compared to that of pCATp (its CAT activity plotted as 100). However, p64(++)CATp, which has two copies of the 64-bp fragment placed in a head-to-tail sense orientation, showed less potentiation than p64(+)(–262/–201)CATp, and the induction caused by one copy of 64 bp in an inverted orientation, p64(–)(–201/–262)CATp, was even lower than that obtained by p64(++)CATp. Moreover, placing the 64-bp fragment downstream of the CAT gene at both orientations not only resulted in the loss of ability to enhance transcription, but also repressed the reporter expression. Therefore, these results suggest that this 64-bp region may function as a position-dependent enhancer and that the upstream position to the basal promoter is required for it to activate a TATA-containing (at least the SV40 case shown herein) promoter activity.

Function of TR2-PACE Is Orientation Dependent on TR2 Basal Promoter

The experiments we have already described, illustrate that the 64-bp fragment (–263/–201) is functioning as a *cis*-regulatory element to augment a promoter containing a TATA box when placed upstream to the promoter. Therefore, we named this 64-bp region the TR2-PACE. We then set forth to examine this entire sequence in depth, to study the mechanism responsible for the *cis*-acting activity. Intrerestingly, we found that there is one SP1-like sequence (SP) located in the 5'-region and a purine-rich sequence (Pu) laying at the center portion of TR2-PACE (Fig.4A). Sequences such as these have been found upstream of many TATA-less and/or house keeping gene promoters, and they have been demonstrated to play key regulatory roles in the expression of their own genes. For example, the promoter of the human androgen receptor gene is one of the typical cases of this type of regulation *(16)*. To clearly define the region responsible for the regulatory function, we used the restriction enzyme *Mnl*I to separate these two regions and tested them individually. Since the promoter of the TR2 gene contains an Inr-element, yet lacks a TATA box, we decided to also test the behavior of TR2-PACE when connected to its own core promoter. For this purpose, we constructed the TR2BCAT plasmid, in which the CAT reporter gene is driven by the TR2 basal promoter (–44/+65), and then cloned the TR2-PACE, SP, or Pu fragment (Fig.4A) upstream in either a sense or an antisense orientation with respect to that of the TR2 basal promoter.

Using the CAT activity of the p-2666TR2CAT as the maximal range control, and the pTR2BCAT as the basal range control, we performed the transient transfection

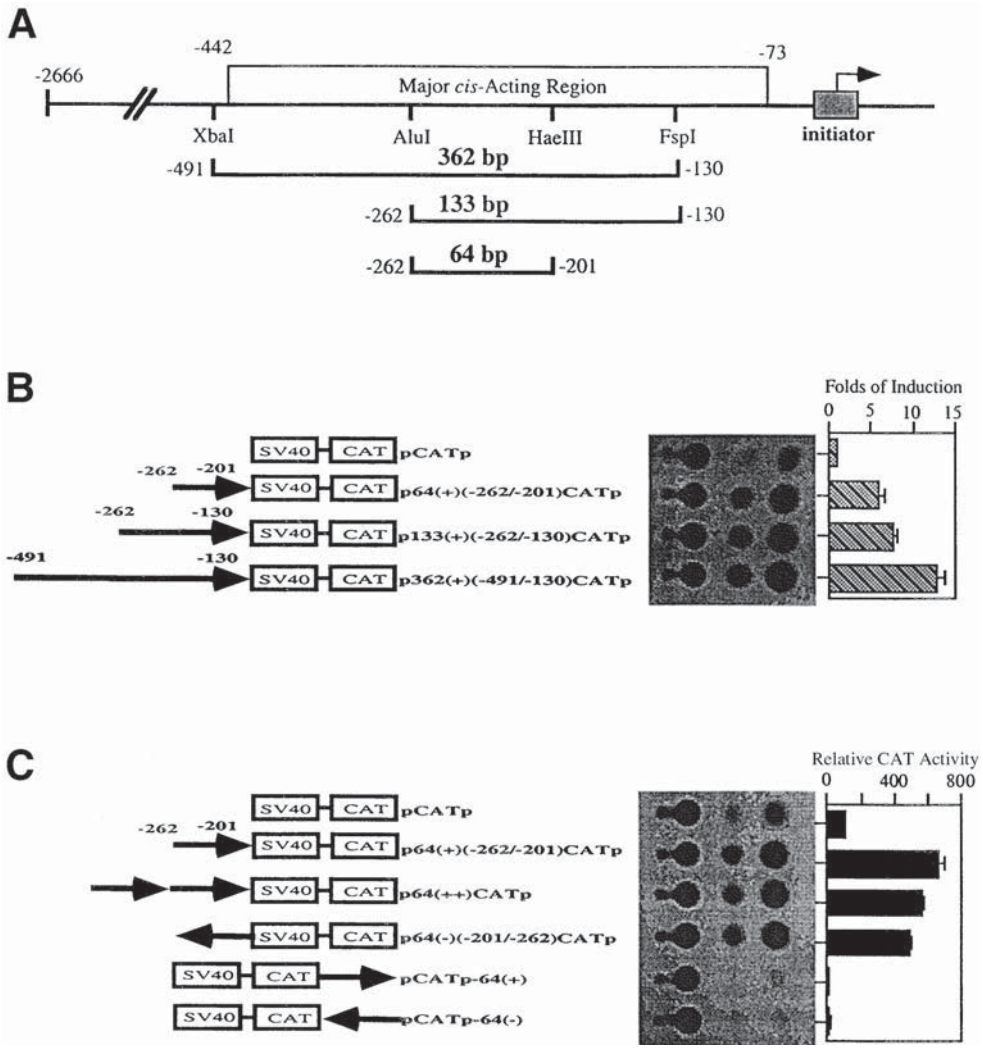

Fig.3. Enhancer activity of the 64-bp region is position dependent. **(A)** Overview of the –441/–73 major *cis*-acting region. The size and the nucleotide numbering are depicted according to the length and position of the regions subcloned upstream or downstream to pCATp (Promega, Madison, WI). **(B)** DNA fragments described in **(A)** were inserted upstream to SV40 minimal promoter-driven CAT gene in the pCATP. The CAT activity (far right, bar graph), after constructs (3 μg) were transiently transfected into PC-3 cells, was measured and normalized with β-galactosidase activity. The CAT activities of all plasmids tested were calculated as the fold of induction with pCATP as one fold. Data points were the mean value ±SE of five separate experiments. **(C)** The 64-bp region was inserted upstream or downstream to the SV40 minimal promoter-driven CAT gene in either a sense or an antisense orientation. The CAT activity (far right, bar graph) was measured and normalized with β-galactosidase activity. The CAT activities of all plasmids tested were calculated as relative CAT activity with pCATP as 100%.

experiments in PC3 cells. The results shown in Fig. 4B revealed that only the CAT activity of p64(+)TR2BCAT (lane 3), which has TR2-PACE placed in a sense orientation, can be induced as high as that of the p-2666TR2CAT (Fig. 4B, lane 1, plotted as 100). Near basal levels of CAT activity were obtained for the rest of the constructs (Fig.4B, pTR2BCAT, lane 2; p64(–)TR2BCAT, lane 4; pSP(+)TR2BCAT, lane 5; pSP(–)TR2BCAT, lane 6; pPu(+)TR2BCAT, lane 7; and pPu[–]TR2BCAT, lane 8). These results suggest that neither the SP region nor the Pu region alone is capable of functioning to enhance the activity of the TR2 promoter, and that the ability to elevate the TR2 minimal promoter activity is orientation dependent. Unexpectedly, we noticed that TR2-PACE in the sense orientation induced the TR2 gene basal promoter activity sixfold, a level close to the maximal activity reached by the full 2.7-kb reporter construct (Fig. 4B, lanes 1 and lane 3).

Sequence Within TR2-PACE Physically Interacts with Nuclear Protein(s)

To define proteins capable of interacting with the TR2-PACE, the DNA fragments with nuclear extracts were used in a magnesium agarose electrophoretic mobility shift assay (EMSA) experiment *(17)*. The TR2-PACE elicited a specific shifted band (pointed out by an arrow in Fig.5A) with both HeLa and LNCaP nuclear protein extracts, respectively. A 50-fold excess of cold TR2-PACE eliminated, by competition, the majority of the density

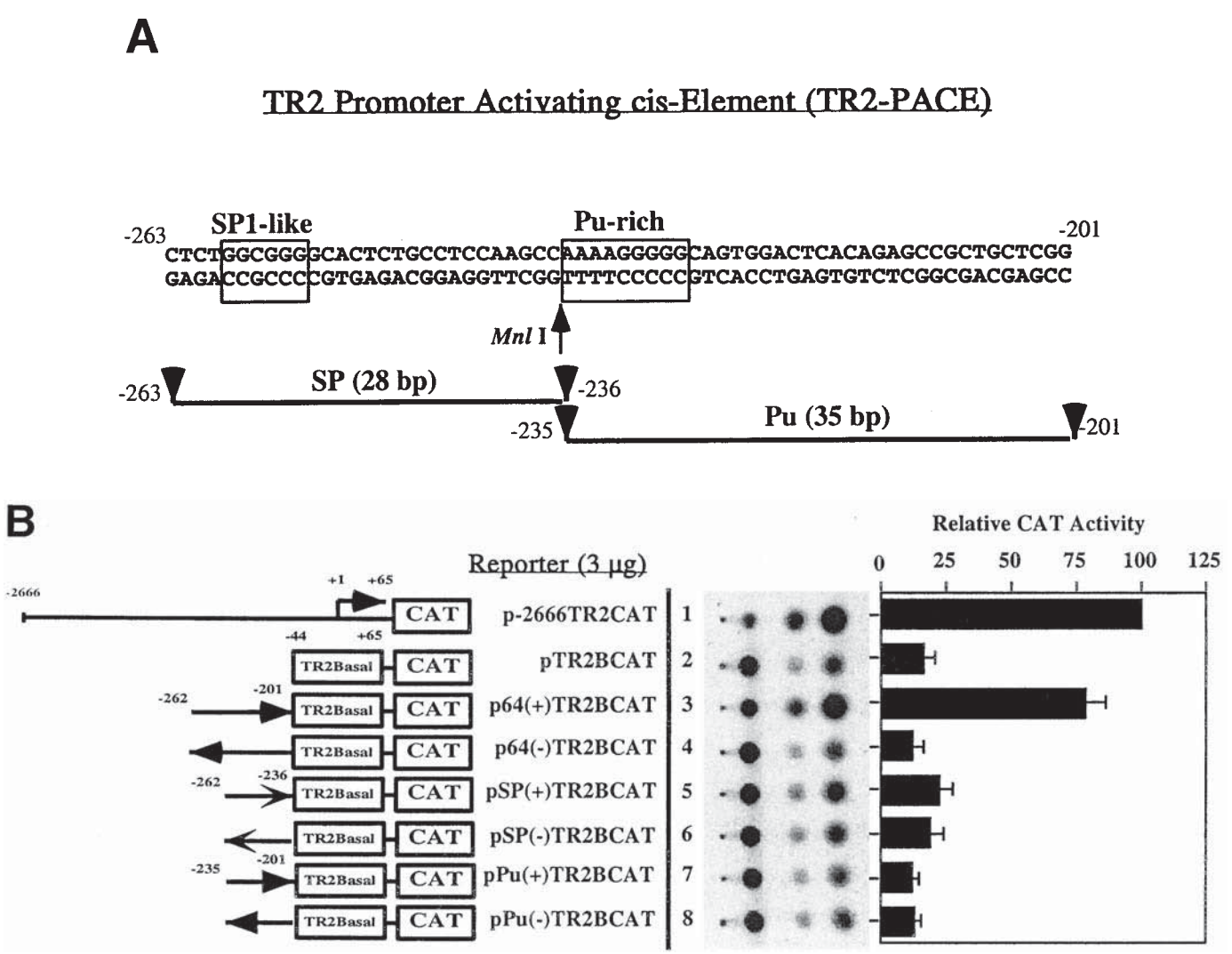

Fig.4. DNA fragments harboring the 64-bp sequence functions as a direction-dependent enhancer to activate TR2 basal promoter. **(A)** Overview of the TR2 promoter activating cis-element (64 bp, –263/–201) (TR2-PACE) locating within the major *cis*-acting region of TR2 5'-flanking sequences. *Mnl*I was the restriction enzyme used to dissect TR2-PACE into two fragments, SP and Pu. **(B)** The 64-bp region was inserted upstream of the TR2 basal promoter-driven CAT gene vector pTR2BCAT in either a sense or an antisense orientation. Obtained from PC-3 cells, the CAT activities (normalized by β-galactosidase activity) of all plasmids tested were calculated as relative CAT activity with p-2666TR2CAT as 100%. Data points were the mean value ± SE of five separate experiments.

of the specific shifted band (Fig. 5A, lanes 3 and 6). Also, to double-check the possibility of any involvement of the transcription factor Sp1 or any potential purine-rich specific binding factors, the use of the anti-Sp1 antibody, which served as an integrator to supershift the DNA protein complex band, showed no detectable change of the shifted position (Fig.5A, lanes 2 and 5). These results suggested that Sp1 transcription factor alone is not involved in TR2-PACE binding. This specific interaction shown in EMSA was further confirmed by Southwestern analysis using HeLa and LNCaP nuclear extracts. Figure 5B shows that a band in both lanes at a position of ~100 kDa of molecular weight (upper arrow) was detected. In addition, a lower molecular weight band (~35 kDa) (Fig. 5B, lower arrow) was also visualized by the binding of TR2-PACE in the lane of LNCaP nuclear extract, suggesting that a common and possibly a cell-type specific nuclear protein can bind to TR2-PACE. Together, a correlation can be demonstrated by both analyses to support our hypothesis that the TR2-PACE is both a novel and a non-classical enhancer.

Discussion

One of the most important steps in regulating gene expression is through transcriptional activation and initiation. Transcriptional activation of eukaryotic genes during development or in response to extracellular signals, involves the regulated assembly of multiple protein complexes on enhancers and core promoters. The complex nature of these processes provides virtually unlimited possibilities for regu-

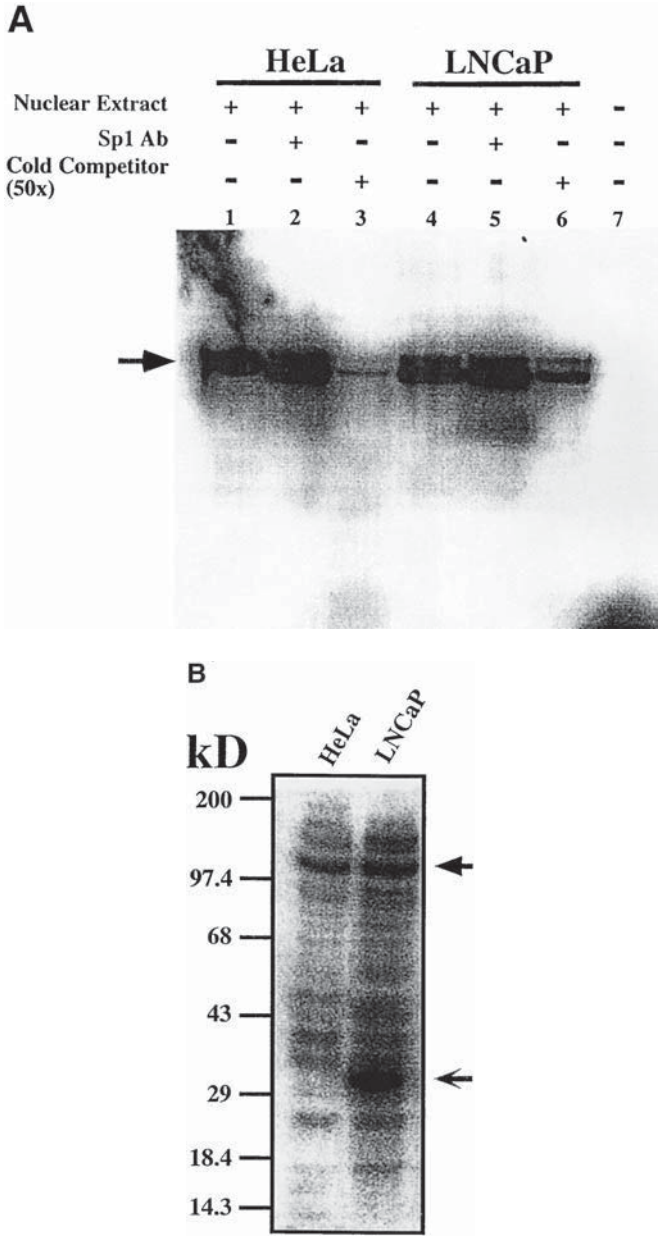

Fig.5. TR2-PACE binds to specific proteins in nuclear protein extract. **(A)** Fifteen micrograms of nuclear protein extracts isolated from human HeLa and LNCaP (prostate cancer cell) were incubated with TR2-PACE and analyzed by an EMSA. In the assay, competition with an excess amount of nonlabeled oligos was performed as indicated each lane. The double-stranded oligonucleotides used in the assay (^{32}P end labelled) and in the cold competitions is TR2-PACE (sequence shown in Fig.4B) at 50 folds higher concentration to the hot probe (lanes 3 and 6). The monoclonal antibody which against Sp1 (Santa Cruz Biotechnology, Santa Cruz) was added to the reaction at lanes 2 and 5. Samples were resolved in a 1.4% magnesium agarose gel (low LEE agarose; Fisher). The arrows indicate the migration-position of the shifting complexes. **(B)** In a Southwestern experiment, two tandem repeats of TR2-PACE were used to probe against sixty μg of HeLa (lane 1) and LNCaP (lane 2) nuclear extract that was separated by 7.5% tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis. Two bands detected are indicated by the upper arrow (~100 kDa) and a lower arrow (~35 kDa).

lation and results in an elaborate fail-safe mechanism for controlling gene expression. Central players in this process are sequence-specific transcription factors that select genes to be activated and orchestrate the assembly of a transcription "machine" at the core promoter and alter the mRNA synthesis *(18)*. Thus, it is essential to map the core promoter of TR2 gene to be able to reveal the nature of its expression regulation. Since there is an Inr sequence (–1/+9) surrounding the cap site of the otherwise TATA-less TR2 gene 5'-flanking region *(5)*, we needed to test the Inr within the TR2 gene to determine whether it behaves as a core promoter. Therefore, we truncated the Inr sequences and assayed for the loss of CAT activity. The results showed that the CAT activity dropped to a level comparable to that of the background activity of the parental vector indicating, evidently, that this specific Inr is serving as a core promoter to initiate gene transcription.

Our earlier study showed that the surrounding sequences near Inr are GC-rich and do not contain a consensus TATAA motif *(5)*. Transcription factor Sp1 is generally involved in and functions as a central player to activate as well as orchestrate the transcription of the GC-rich and TATA-less promoters by RNA polymerase II *(19)*. This happens in a wide variety of constitutively expressed cellular genes, including the human low-density lipoprotein receptor *(20)*, retinoblastoma gene product *(21)*, and insulin receptor *(22)*. It has been demonstrated that Sp1, Inr, and TFIID are adequate to initiate transcription from TATA-less promoters *(23)*. Whether Sp1, especially when there is a consensus Sp1 binding site residing at the 5' of the TR2-PACE, plays any significant role in the expression of the TR2 gene needs to be determined.

Recent studies have shown that the activity of at least some natural enhancers requires the assembly of a stereospecific nucleoprotein complex for achieving a high level of specificity and gene expression. Changes in the relative positions or orientations of the stereospecifc protein binding sites may lead to the inactivation of the enhancer *(24)*. In the model system of TATA-containing SV40 promoter (Fig.3C), TR2-PACE insertion at an upstream position elicited induction of CAT activity, although the level of the activity differed depending on the orientation. However, altering the insertion to the downstream position not only yielded a loss of induction but conversely generated a repressing effect. When switching the model system to a promoter lacking the TATA box, inverting the TR2-PACE or inserting it at a position upstream of the TR2 basal promoter was able to diminish activation.

Similar phenomena have been observed in other enhancers that are recognized by the transcription factor Yin-Yang 1 (YY1) *(25)*. YY1 is an ubiquitously expressed zinc finger DNA-binding protein. It can also act as a component of the basal transcription complex when binding at the Inr element *(26)*. As a typical example, YY1 can either activate or repress the c-Fos promoter based on the orientation or the

position of a YY1 binding site relative to the promoter *(25)*. The main function of YY1 to this promoter is to bend DNA to facilitate the protein-protein interaction *(25)*. Proteins sharing this characteristic have been proposed to serve as "architectural" elements in the modulation of intrinsic enhancer architecture. Participation of those factors results in the formation of an enhancer-promoter orientation that favors protein-protein interactions into a "functional" nucleoprotein complex called the *enhancersome (27,28)*. However, since there is no YY1 binding site within TR2-PACE, whether the nuclear proteins detected in Southwestern analysis may share a nature similar to YY1, in order to interact with TR2-PACE, is an intriguing possibility that remains to be determined.

In CAT reporter assays (Fig.4B, lanes 5–8), EMSA (Fig.5A), and Southwestern analysis (Fig.5B), the results argue clearly against an independent mechanism of either the SP1-like or the purine-rich sequences to be responsible for the function of TR2-PACE. Comparing the reporter response of TR2BCAT, the CAT activity of SP1-like or purine-rich region shows no sign of enhancer capacity. Taken at face value, neither the SP nor Pu can be dissected out from a functional TR2-PACE. Also, in a nice agreement with the results from the CAT assays, there was no ability to further slow down the migration of the shifted bands by anti-Sp1 antibody. However, it may be premature to exclude that other member of the Sp family *(29)* or a Pu-binding protein may participate as a component of a big regulatory protein complex to be responsible for the function of the TR2-PACE. The fact that the nuclear protein-TR2-PACE complex requires a 1.4% magnesium agarose EMSA to be resolved, virtually indicates a relatively large complex. We believe this suggests there may be multiple molecules of high molecular weight DNA-binding proteins involved in the function of TR2-PACE.

In situ hybridization *(6)* determined TR2 expression to be extensive during fetal development, and in adult rat tissues the TR2 is expressed with higher abundance in male reproductive organs *(3)*. Based on the Southwestern data presented, a mechanistic theory can be made for these findings. Using the TR2-PACE probe, we detected an ~100-kDa nuclear protein in both HeLa and LNCaP cells, and a ~35-kDa nuclear protein in LNCaP cells alone (Fig. 5B). These data suggest that the larger protein (~100 kDa) may be responsible for the extensive expression of TR2 during development, whereas the ~35 kDa protein may be responsible for the selective expression of TR2 in the adult stage.

In conclusion, we demonstrated in this report that the *cis*-acting regulatory region of the TR2 gene can be narrowed down to 64-bp (–263/–201), which we called TR2-PACE. The CAT reporter activity was enhanced significantly when we placed TR2-PACE in the sense orientation upstream of a basal promoter containing either a TATA box or an Inr fused to a reporter gene. Both EMSA and Southwestern data showed that certain unidentified nuclear protein(s) specifically bind to this TR2-PACE. In sum, the evidence clearly suggests that the TR2-PACE may function as an orientation- and position-dependent *cis*-activating element. Together with its potential *trans*-acting nuclear factors, TR2-PACE may play an essential role in TR2 gene expression.

Materials and Methods

Construction of Plasmids

A series of deletion mutants of human TR2 gene promoter CAT plasmids (p-2180TR2CAT, p-1857TR2CAT, p-1714TR2CAT, p-1390TR2CAT, p-1354TR2CAT, p-959TR2CAT, p-441TR2CAT, p-242TR2CAT, p-73TR2 CAT, p-38TR2CAT, p+6TR2CAT) were created from p-2666TR2CAT using Erase-a-Base System (Promega). Another series of internal deletion mutants of human TR2 gene promoter CAT plasmids (p-2666TR2CAT) were constructed using three-way ligation. The *Xba*I-*Pst*I-digested p-2666CAT served as the vector in ligation with 200 bp of *Fsp*I-*Pst*I to create Δ358; 140 bp of *Xba*I-*Hinc*II and 200 bp of *Fsp*I-*Pst*I to create Δ226; 230 bp of *Xba*I-*Alu*I and 200 bp of *Fsp*I-*Pst*I to create Δ135; 350 of bp *Xba*I-*Hae*III and 200 bp of *Fsp*I-*Pst*I to create Δ71; 140 bp of *Xba*I-*Hinc*II and 330 bp of *Afl*III-*Pst*I to create Δ91; and, 230 bp of *Xba*I-*Alu*I and 270 bp of *Hae*III-*Pst*I to create Δ64 (Fig. 2 shows the relative restriction positions). Blunt-end fragments were inserted into pCATp (Promega) at a blunt-ended *Bgl*II site for the upstream position or at a blunt-ended *Xba*I site for the downstream position of CAT gene. Insertion of 64-bp of *Alu*I-*Hae*III created p64(+)(–262/–201)CATp, p64(++)CATp, p64(–)(–201/–262)CATp, pCATp-64(+), and pCATp-64(–). Insertion of 133 bp of *Alu*I-*Fsp*I created p133(+)(–262/–130)CATp. Insertion of 362 bp of *Xba*I-*Fsp*I created p362(+)(–491/–130)CATp (*see* Fig.3). Ligation of the 120-bp *Alu* I-*Pst* I with *Sma* I-*Pst* I digested pBSCAT2 produced the pTR2BCAT. Subsequently, the 64-bp TR2-PACE, *Mnl*I-digested 28-bp SP, and 35-bp Pu (*see* Fig.4B) were then inserted upstream of TR2 basal promoter with both orientations to create p64(+)TR2BCAT, p64(–)TR2BCAT, pSP(+)TR2BCAT, pSP(–)TR2BCAT, pPu(+)TR2BCAT, and pPu(–)TR2BCAT (*see* Fig.4C). DNA sequencing was then conducted on each plasmid construct to confirm the accuracy via the dideoxy chain termination method *(9)* with T7 Sequenase (USB, Amersham), and electrophoresed in denatured sequencing gel (National Diagnostics).

Cell Culture, Transfection, and CAT Assay

Cells were cultured in Dulbecco's modified Eagle's F-12 medium supplemented with 5% fetal calf serum, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin (Sigma, St. Louis, MO). For transfection studies, cells were then transfected with 3 μg of the reporter plasmid and 6 μg of pBluescript plasmid (Promega) using the calcium phosphate method described previously *(9,10)*. β-Galactosidase expression plasmids

(1 μg) were cotransfected as an internal control to normalize the transfection efficiency. After 24 h of transfection, the medium was changed once and the cells were cultured for another 24 h, and then harvested. Next, the cells were lysed in 250 m*M* Tris-HCl (pH 7.8) by freeze-thawing, and the resulting supernatants were assayed for CAT activity. The reaction product was extracted with ethyl acetate (Mallinckrodt), and then applied to a thin-layer chromatography plate (Sigma) and developed in a 95% chloroform: 5% methanol solvent. The CAT activity was quantitated by ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Preparation of Nuclear Lysate

Nuclear protein extracts from HeLa and LNCaP cells were harvested after being washed twice in phosphate buffered saline buffer. To prepare nuclei, cells were resuspended in hypotonic buffer (10 m*M* HEPES-KOH, pH 7.9; 1.5 m*M* $MgCl_2$; 10 m*M* KCl; 0.1% NP40) and incubated on ice for 10 min. Nuclei were precipitated with 3000*g* centrifugation at 4°C for 10 min. After washing once with hypotonic buffer, the nuclei were lysed in lysis buffer (50 m*M* Tris-HCl, pH 8.0; 150 m*M* NaCl; 1% Triton X-100) and incubated on ice for 30 min. The nuclear lysates were precleared by 20,000*g* centrifugation at 4°C for 15 min. Protein concentration was measured by Bradford assays.

EMSA and Southwestern Analysis

Oligonucleotides TR2-PACE (sequence shown in Fig.4B) for the EMSA were end labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs, Beverly, MA). The anti-Sp1 antibody was purchased from Santa Cruz Biotechnology. Approximately 1×10^5 cpm of DNA were used in each reaction with 15 μg of nuclear protein. Samples were resolved in a 1.4% magnesium agarose gel (low LEE agarose; Fisher) and electrophoresed in 1X magnesium agarose gel buffer (44.5 m*M* Tris-base, 44.5 m*M,* boric acid and 7.5 m*M* magnesium acetate) at room temperature. Two copies of the 64-bp TR2-PACE (–263/–201) were inserted in a sense orientation in the *Eco*RV site of pBluescript SK(+) (Stratagene) to generate pBS64-2. The probe for Southwestern analysis *(30)*, about 144-bp in size, was obtained from pBS64-2 by *Eco*RI/*Hind*III digestion and ^{32}P end labeled. Approximately 1×10^5 cpm of DNA were used per Southwestern experiment.

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