The key expression of the simian virus 40 (SV40) major late promoter could be repressed by the human TR4 orphan receptor via the +55 region of the SV40 major late promoter (nucleotide numbers 368–389, 5'-GTATTAGGTTCTAGGTATGGCATGA-3'). Using the coupled in vitro transcription and translated TR4 orphan receptor with a molecular mass of 67.3 kilodaltons, electrophoretic mobility shift assay showed specific binding with a dissociation constant of 1.09 nM between the TR4 orphan receptor and the SV40 +55 oligonucleotides. In addition, chloramphenicol acetyltransferase assay demonstrated that this SV40 +55 region can function as a repressor via the TR4 orphan receptor, suppressing the transcriptional activities of both SV40 early and late promoters. Together, our data suggest that the TR4 orphan receptor may play an important role for the suppression of the SV40 gene expression.

Steroid receptors regulate the transcription of complex gene networks and subsequently control diverse aspects of growth, development, and differentiation (1, 2). The steroid receptor superfamily includes receptors for steroid hormones, thyroid hormones, retinoids, and a large number of orphan receptors whose cognate ligands have not been identified. These steroid receptors acting as transcriptional factors bind to specific DNA sequences, hormone response elements (HREs), and thereby regulate their target genes. In short, the HRE is composed of 6 base pairs forming the core recognition motif. The palindromic half-site sequence AGAAC is preferentially recognized by the receptors for androgen, glucocorticoid, mineralocorticoid, and progesterone. In contrast, estrogen, thyroid, retinoic acid, retinoid X, vitamin D, and many orphan receptors preferentially bind to repeats of a half-site sequence AGGTCA. However, a number of orphan receptors can be classified into a third group of monomeric receptors which apparently bind as monomers to a single half-site of the sequence AGGTCA preceded by a short AT-rich sequence (2).

We have isolated human and rat TR4 orphan receptor cDNAs from the hypothalamic supraoptic nucleus, prostate, and testis by degenerative polymerase chain reaction cloning (3). The open reading frame of human TR4 orphan receptor cDNA encodes a polypeptide of 615 amino acids with a calculated molecular mass of 67.3 kilodaltons. In the 3'-untranslated region of the TR4 orphan receptor, an eukaryotic polyadenylation signal ATTAAA is present between the nucleotide numbers 2222 and 2227. Human TR4 orphan receptor shows a high degree of nucleotide sequence homology with the TR2-11 orphan receptor (4, 5). The homology between these two orphan receptors in N-terminal, DNA-binding and C-terminal domains is 51, 81, and 65%, respectively (3). The high homology between the TR2 and TR4 orphan receptors suggests that these two orphan receptors constitute an unique subfamily within the steroid receptor superfamily (3). Recently, the TR4 orphan receptor has also been identified from human lymphoblastoma cells (named as TAK1), and the expression in a variety of tissues has been demonstrated by Northern blot analysis and in situ hybridization (6). Most tissues contained TR4 transcripts at markedly variable levels. Thus, the TR4 orphan receptor may play some roles in the regulation of gene expression in specific cell types. Very recently, a DNA binding site for orphan receptors in the SV40 major late promoter (MLP) (7–9, 27) has been demonstrated to be a natural HRE for the TR2 orphan receptor (10). Since both TR2 and TR4 orphan receptors are structurally related, we therefore set out to investigate the interaction between the transcriptional initiation site of the SV40-MLP and the TR4 orphan receptor. Various truncations of the TR4 orphan receptor were also generated to identify functional domains within the TR4 orphan receptor. Results from DNA binding studies and transfectional assays suggested that the TR4 orphan receptor can function as a suppressor for gene expression of the SV40. This may further expand the roles of the TR4 orphan receptor in the transcriptional regulation of viruses.

MATERIALS AND METHODS

Plasmid Construction—For coupled in vitro transcription and translation, the full-length coding sequence of the TR4 orphan receptor cDNA was cloned into either the pCMX vector (pCMX-TR4) (kindly provided by Dr. R. M. Evans) or the pET14b vector (pET14b-TR4) (Novagen) (3). The N-terminal truncated TR4 orphan receptor, pET14b-TR4C plasmid, was generated from the pCMX-TR4 by polymerase chain reaction amplification using two primers (5'-TCCAGCGGCAACAGGCTGAA-3' and 5'-CTATAGACTGACACCGGCTGACGTA-3') to cover the entire DNA-binding and C-terminal domains. The C-terminal truncated TR4 orphan receptor, pET14b-TR4S plasmid, was derived from a deletion of the region right after HindIII site of the pET14b-TR4. Plasmids pBL-SVL-CAT, pBL-SV40RE-CAT, pSV55wt1, and pSV55 mut1 were described previously (10).

Coupled In Vitro Transcription and Translation—Plasmids containing the full-length or truncated TR4 orphan receptor cDNAs were in
Suppression of SV40-MLP by TR4 Orphan Receptor

RESULTS

TR4 Orphan Receptor Binds Specifically to the +55 Region of the SV40-MLP—The TR4 orphan receptor shows a higher degree of nucleotide sequence homology with the TR2 orphan receptor (3), but any functional activity of this novel orphan receptor is unknown. One of the initiator-binding sites of the SV40-MLP consists of a core recognition motif AGGTCA half-site (7–9, 27), which has been demonstrated as a natural HRE for the TR2 orphan receptor (10). We suspected that the TR4 orphan receptor might behave similar to the TR2 orphan receptor based on the structure-function relationship within this unique subclass of the steroid receptor superfamily. To test this hypothesis, we prepared in vitro expressed TR4 orphan receptor for EMSA and DNA-protein binding assay. A cDNA encoding the intact TR4 orphan receptor was in vitro transcribed and translated to produce a protein of the expected molecular mass of 67.3 kilodaltons as shown in Fig. 1A. In contrast, the mock-translated control expressed no detectable product (lane 2). To investigate the possible interaction between the TR4 orphan receptor and the +55 region of the SV40-MLP, double-stranded oligonucleotides corresponding to the SV40 nucleotide numbers 368–389 were used for the EMSA (11). A specific DNA-protein complex was visualized using the probe and the in vitro expressed TR4 orphan receptor in the EMSA as shown in Fig. 1B (lane 2). This radioactive DNA-protein complex could be abolished in the presence of 100-fold molar excesses of unlabeled oligonucleotides (lane 3), but remained intact in the presence of mutant SV40 +55 oligonucleotides (lane 4). Moreover, there was no specific interaction between the probe and the mock-translated product (lane 5). These data suggested that the TR4 orphan receptor indeed has the ability to specifically bind to the +55 region of the SV40-MLP and form a single complex with this DNA element.

In order to determine the DNA protein binding affinity between the TR4 orphan receptor and the SV40 +55 region in more detail, we performed Scatchard binding analysis by the EMSA (Fig. 2). Constant amounts of in vitro expressed TR4 orphan receptor (60 ng) were incubated with different concentrations of the SV40 +55 probe (0.025–12.8 ng). DNA-protein complexes were resolved in the EMSA (Fig. 2A). Scatchard plot analysis revealed a single binding component for the specific DNA-protein complex with a dissociation constant ($K_d$) of 1.09

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was carried out as described previously (10). In general, the double-stranded SV40 +55 oligonucleotides corresponding to SV40 nucleotides 368–389 (5'-GTTCAGGGTCAGGTCAGAAA) were end-labeled as a probe (11). The mutant SV40 +55 oligonucleotides contain two complementary sequences (5'-CGTTAAAGGGTCAGGTCAGAAA). For competition reactions, cold double-stranded oligonucleotides were mixed with the labeled probe prior to the addition to the reactions. For antibody supershift analysis, $\mu$g of the monoclonal anti-TR4 orphan receptor antibody (G252-303.4) was added into the reactions for 15 min at room temperature prior to loading on a gel. Hybridoma cells were cultured in the RPMI medium 1640 (Life Technologies, Inc.).

DNA-Protein Binding Assay—DNA-protein binding assay was performed as described previously (10) with modifications that will be described elsewhere. The free probe and DNA-protein complexes resolved by EMSA were quantified by PhosphorImager (Molecular Dynamics). The dissociation constant ($K_d$) value was determined from the minus reciprocal of the slope of the line generated from the empirical data. Transfection Experiments—HeLa cell culture and transfection procedures were described previously (10, 12–14). To normalize the transfection efficiency, the $\beta$-galactosidase expression vector was co-transfected (12–14).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{Binding of the in vitro expressed TR4 orphan receptor to the +55 region of the SV40-MLP. A, analysis of in vitro translated TR4 orphan receptor in SDS-12% polyacrylamide gel electrophoresis. Lane 1 displays $^{35}$S-labeled protein standards. The mock-translated product and the TR4 orphan receptor expressed in a coupled in vitro transcription-translation system are shown in lanes 2 and 3, respectively. The product of the TR4 orphan receptor is indicated on the right with an expected molecular mass of 67.3 kilodaltons. The minor products probably arise from internal initiation of translation or limited degradation. B, binding of the TR4 orphan receptor to the +55 region of the SV40-MLP. EMSA was performed with the in vitro expressed TR4 orphan receptor and the $^{32}$P-end-labeled probe. Lane 1 shows the probe alone. Binding reaction mixtures incubated with the probe and the in vitro synthesized TR4 orphan receptor (lane 2), in the presence of 100-fold molar excesses of unlabeled wild-type (wt) oligonucleotides (lane 3) or mutant (mut) oligonucleotides (lane 4) are shown. Lane 5 displays binding reaction mixtures incubated with mock-translated product and the probe. The retarded complex is indicated by the arrowhead, whereas nonspecific complexes appear between the retarded band and the free probe at the bottom.}
\end{figure}

\textsuperscript{2} Y. Lee and C. Chang, manuscript in preparation.
Interestingly, we only detected the original antibody (lanes 12-15) a complex which could be supershifted in the presence of the co-transfection of mammalian expression vectors and CAT polyacylaminolase gel electrophoresis. Six points of experimental data are shown here. A, 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 Enzyme program (Biosoft).

TR4 orphan receptor antibody (G232-303.4) recognizing the N-terminal region of the TR4 orphan receptor (amino acid residues 7–136) could further supershift this DNA-protein complex (indicated by the arrowhead) and the free probe were quantified by Phosphorimagery (Molecular Dynamics). Six points of experimental data are shown here. 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).

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The C-terminal truncation might also form another smaller DNA-protein complex which co-migrated with one of the nonspecific complexes (lanes 8-10, small arrowhead). As expected, this smaller complex could not be supershifted by the monoclonal antibody (lane 11). The C-terminal truncation might also form a complex which could be supershifted in the presence of the antibody (lanes 12-15). Interestingly, we only detected the original DNA-protein complexes when either the N- or C-terminal truncations was incubated with the intact TR4 orphan receptor and the DNA probe (lanes 16-23). Taken together, these results suggested that the TR4 orphan receptor and its variants may individually recognize the +55 region of the SV40-MLP, and no data suggest the heterodimerization may happen between the intact and truncated TR4 orphan receptors on this DNA element.

Repression of the Gene Expression of the SV40-MLP by the TR4 Orphan Receptor—Thus far, all of our data supported the idea that the TR4 orphan receptor may specifically bind to the +55 region of the SV40-MLP. To examine whether the TR4 orphan receptor can play any functional role in the SV40 gene expression via interaction with the SV40 +55 region, we employed the chloramphenicol acetyltransferase (CAT) assay with the co-transfection of mammalian expression vectors and CAT reporter plasmids into HeLa cells. As shown in Fig. 4, the co-transfection of expression vector containing the full-length TR4 orphan receptor cDNA (pCMX-TR4) and either pBL-SVL-CAT (without the SV40 +55 region) or pBL-SVLRE-CAT (with the SV40 +55 region) reporter plasmids indicated that the TR4 orphan receptor cannot affect the transcriptional activity of the SV40-MLP without the +55 region. In the presence of the +55 region, the TR4 orphan receptor repressed the transcriptional activity of the SV40-MLP. This result suggested that the TR4 orphan receptor may function as a repressor for the transcriptional activity of the SV40-MLP by binding to the SV40 +55 region. This finding is in agreement with previous results in our study of the TR2 orphan receptor (10). Both the TR2 and
TR4 orphan receptors can function as repressors for the gene expression of the SV40-MLP.

Because our data demonstrated that the TR4 orphan receptor may suppress the SV40 gene expression, we tried to determine if the TR4 orphan receptor needs activator(s) for the activation of its repression. In order to test this hypothesis, we used regular fetal bovine serum (FBS) versus charcoal-treated FBS (CTS) in the transient transfection experiments. As shown in Fig. 5, the repressor function of the TR4 orphan receptor was dependent on the presence of a potential activator(s) in the serum. In contrast, charcoal can eliminate such a repressor(s) by the removal of the potential TR4 activator(s) from the serum. These results further indicated that this activator-dependent repression of the SV40 gene expression may rely on the interaction between the TR4 orphan receptor and the +55 region. As expected, the mutant +55 region had no influence on the repression of the SV40 gene expression in the presence of either FBS or CTS.

**DISCUSSION**

In the present study, we have demonstrated that the human TR4 orphan receptor may suppress gene expression of the SV40-MLP. Some orphan receptors have been shown to affect gene expression of viruses. For example, we have previously identified that human TR2 orphan receptor, a closely related subclass member to the TR4 orphan receptor, can bind and repress transcriptional initiation from the +55 region of the SV40-MLP (10). Another orphan receptor (TR3) identified in our laboratory was also able to induce the transcriptional activation of its repression. In order to test this hypothesis, we used regular fetal bovine serum (FBS) versus charcoal-treated FBS (CTS) in the transient transfection experiments. As shown in Fig. 5, the repressor function of the TR4 orphan receptor was dependent on the presence of a potential activator(s) in the serum. In contrast, charcoal can eliminate such a repressor(s) by the removal of the potential TR4 activator(s) from the serum. These results further indicated that this activator-dependent repression of the SV40 gene expression may rely on the interaction between the TR4 orphan receptor and the +55 region. As expected, the mutant +55 region had no influence on the repression of the SV40 gene expression in the presence of either FBS or CTS.

transcriptional inactivation (Ti) domain, and hepad repeats at the C-terminal region, polarity properties, and ligand specificity of nuclear receptors contribute to the recognition of DNA response elements (2). Moreover, nuclear accessory factor and even nuclear matrix three-dimensional structure also influence these DNA-protein interactions (2, 22).

Monomeric, homodimeric, and heterodimeric modes of DNA binding result from receptor-specific differences in the DNA binding and dimerization domains (2). It has been documented that the C-terminal hepad repeats which are structurally similar to the leucine zipper dimerization domains are involved in the dimerization of thyroid hormone and retinoic acid receptors (23–26). However, we were unable to demonstrate homo- or heterodimer formation using the intact and truncated TR4 orphan receptors by EMSA in the present study. Whether the TR4 orphan receptor has the ability to form monomeric, dimeric, or both modes of DNA binding may remain an interesting puzzle to be solved.

Most recent evidence suggests that both ligands and phosphorylation play important roles in activation of steroid receptors (16). Many orphan receptors have been discovered by cross-hybridization with known steroid receptor cDNAs. Consequently, these orphan receptors have unknown ligands (or do not need ligands to be activated) and usually unknown physiological function (16). Therefore, identification of ligands for these orphan receptors remains a key to understand the possible roles of these transcriptional regulators. In this current study, we found that TR4 orphan receptor requires a potential activator(s) in the serum to be activated. This activator-dependent suppression of the SV40 gene expression by TR4 orphan receptor can be eliminated in the presence of CTS. Further characterization of this activator(s) will be the next important step to follow.

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Suppression of Gene Expression on the Simian Virus 40 Major Late Promoter by Human TR4 Orphan Receptor: A MEMBER OF THE STEROID RECEPTOR SUPERFAMILY
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