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# Direct Modulation of Simian Virus 40 Late Gene Expression by Thyroid Hormone and Its Receptor

FENGRONG ZUO,[1]† RICHARD J. KRAUS,[1] TOD GULICK,[2]‡ DAVID D. MOORE,[2] AND JANET E. MERTZ[1]*

*McArdle Laboratory For Cancer Research, University of Wisconsin Medical School, Madison, Wisconsin 53706-1599,*[1] *and Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114*[2]

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**Transcription of the late genes of simian virus 40 (SV40) is repressed during the early phase of the lytic cycle of infection of primate cells by the binding of cellular factors, called IBP-s, to the SV40 late promoter; repression is relieved after the onset of viral DNA replication by titration of these repressors (S. R. Wiley, R. J. Kraus, F. R. Zuo, E. E. Murray, K. Loritz, and J. E. Mertz, Genes Dev. 7:2206–2219, 1993). Recently, we showed that IBP-s consists of several members of the steroid/thyroid hormone receptor superfamily (F. Zuo and J. E. Mertz, Proc. Natl. Acad. Sci. USA 92:8586–8590, 1995). Here, we show that the thyroid hormone receptor TRα1, in combination with retinoid X receptor α (RXRα), is specifically bound at the transcriptional initiation site of the major late promoter of SV40. This binding repressed transcription from the SV40 late promoter by preventing the formation of pre-initiation complexes. Addition of the thyroid hormone 3,5,3′-L-triiodothyronine ($T_3$) resulted in reversal of this repression in cotransfected CV-1 cells. Interestingly, repression did not occur when this thyroid response element (TRE) was translocated to 50 bp upstream of the major late initiation site. Binding of TRα1/RXRα heterodimers to this TRE induced bending of the promoter DNA. We conclude that hormones and their receptors can directly affect the expression of SV40, probably by affecting protein-protein and protein-DNA interactions involved in the formation of functional preinitiation complexes.**

Hormones have been known for a long time to affect viral gene expression (for examples, see references 1, 5, 56, and 62). However, few of these studies determined whether the observed effects of hormones on viral gene expression occur via (i) direct interactions of hormones and their receptors with the transcriptional promoters present on the genomes of these viruses or (ii) indirect cross-talk through signal transduction pathways. One exception has been the long-term, elegant studies of Yamamoto and his colleagues showing that regulation of murine mammary tumor virus by glucocorticoids involves direct binding of the glucocorticoid receptor to the promoter in the proviral DNA of this virus (59). Recently, binding sites for several members of the steroid/thyroid hormone receptor superfamily (for recent reviews, see references 51, 52, and 73), including receptors for the thyroid hormone 3,5,3′-L-triiodothyronine ($T_3$), have been identified in the promoters of a number of viruses (for examples, see references 15, 21, 24, 55, 58, 74, 77, and 85). These findings indicate that, at least in some cases, the effects of hormones on viral gene expression may involve direct mechanisms.

The thyroid hormone receptors (TRs) are members of the steroid/thyroid hormone receptor superfamily that bind the thyroid hormone $T_3$. These mammalian receptors are encoded by two genes, each of whose transcripts are alternatively spliced to encode multiple isoforms (for a review, see reference 43). They bind in the nucleus to specific DNA sequences, called thyroid response elements (TREs), found in a variety of cellular (for examples, see references 13, 20, 27, 36, 41, and 78) as well as viral (for examples, see references 21 and 58) genes. The most frequently observed TREs consist of two copies of a hexameric sequence resembling the consensus half-site sequence, 5′-AGGTCA-3′, arranged as direct repeats separated by a 4-bp spacer (DR4); however, other arrangements of the repeats are also possible (26, 52). In most cases, unliganded thyroid receptors can bind TREs, resulting in repression of transcription of the target gene (for examples, see references 7 and 67). However, the binding of $T_3$ to TRs can lead to either activation or repression of transcription depending upon the nature of the TRE and its context within the promoter (for reviews, see references 43 and 52).

TRs can exist in monomeric, homodimeric, and heterodimeric forms (44). Whereas DNA binding and transcriptional activation are enhanced by heterodimerization with retinoid X receptors (RXRs) (reviewed in references 26 and 51), TR/TR homodimers formed on DR4 TREs disassociate in the presence of $T_3$ (for examples, see references 54, 61, and 81). Thus, TR/RXR heterodimers are probably the major functional form in vivo.

Plausible hypotheses to explain transcriptional repression by TRs include (i) passive repression via competition with transcriptional activators or general transcription factors (GTFs) for binding to the same or overlapping sites on the promoter (13, 28) and (ii) active repression via direct or indirect interaction of TR with components of the general transcriptional machinery (3, 14, 22, 23, 35, 42, 66). TR-induced bending of the promoter DNA has also been hypothesized as a mechanism for transcriptional regulation of thyroid hormone-responsive genes by TR (49).

Previously, we had shown that transcription of the late genes of simian virus 40 (SV40) is repressed during the early phase of the lytic cycle of infection by the binding of cellular repressors, collectively called IBP-s, to the SV40 late promoter; this repression is relieved after the onset of viral DNA replication by titration of these repressors (77). Biochemical characterization of IBP-s (77, 85) revealed that it consists, in large part, of the human estrogen-related receptor α1 (25, 37, 80) and the human chicken ovalbumin upstream promoter transcription fac-

* Corresponding author. Phone: (608) 262-2383. Fax: (608) 262-2824. E-mail: mertz@oncology.wisc.edu.

† Present address: Department of Immunology and Rheumatology, Stanford University Medical School, Stanford, CA 94305.

‡ Present address: Diabetes Research Laboratory, Massachusetts General Hospital, Charlestown, MA 02129.

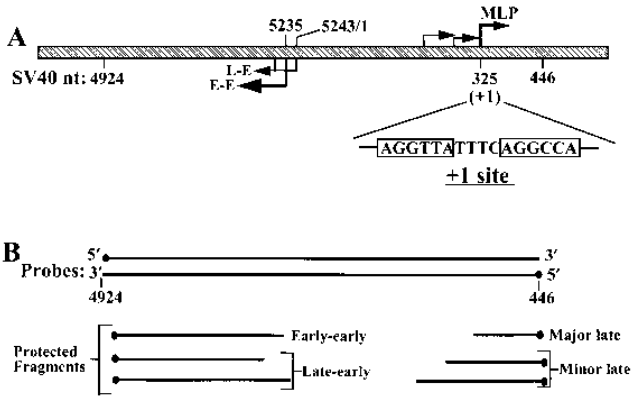


FIG. 1. Bidirectional promoter region of SV40. (A) Schematic diagram of the promoter region of the SV40 genome. The nucleotide numbers are given in the SV40 number system of Buchman et al. (9). The arrows indicate the direction of synthesis and 5′ ends of the early-early (E-E), late-early (L-E), prominent minor late, and major late (ML) transcripts. The sequence of the potential +1 site TRE overlapping the major late initiation site at nt 325 is shown; the boxes indicate the locations of the half-site sequences for binding of TRs to this putative DR4 element. (B) Structures of the probes used in the S1 nuclease mapping experiment shown in Fig. 4 and the fragments protected by hybridization with each of the major and prominent minor SV40 early and late RNA species.

tors 1 and 2 (75, 76), members of the steroid/thyroid hormone receptor superfamily for which ligands are not yet known or do not exist (i.e., orphan receptors). This finding suggested that some members of the steroid/thyroid hormone receptor superfamily may play major, direct roles in the control of the early-to-late switch in regulation of SV40 gene expression. Unfortunately, it is not possible to test the effects of ligands of orphan receptors on SV40 gene expression.

Recently, we discovered that the thyroid hormone receptor α1 (TRα1) in combination with the retinoid X receptor α (RXRα) can also specifically bind with high affinity to a sequence located directly over the initiation site of the SV40 major late promoter (SV40-MLP) (Fig. 1A) (85). Since the natural ligands of TRα1 (65) and RXRα (34, 48) are known, this finding made it possible to test directly the effects of hormones on SV40 gene expression. We show here the following: (i) TRα1/RXRα represses transcription from the SV40 late promoter in the absence of the thyroid hormone $T_3$ both in vitro and in vivo, (ii) overexpression of TRα1 in CV-1 cells results in a delay in the early-to-late switch in SV40 gene expression in vivo, and (iii) the presence of $T_3$ relieves repression by these receptors in cotransfected CV-1 cells. These findings suggest that TR and other nuclear hormone receptors that can bind the SV40-MLP can confer hormonal regulation of the SV40 early-to-late switch in cells that express these receptors. We also show that TRα1/RXRα heterodimers repress transcription from the SV40 late promoter in a context-dependent manner by preventing the formation of functional preinitiation complexes (preICs).

## MATERIALS AND METHODS

**Oligonucleotides and plasmids.** Oligonucleotides were synthesized (Oligos ETC., Inc.), annealed, and gel purified following standard protocols (64). Plasmids pCDMα1, encoding human thyroid receptor α1 (hTRα1) (a gift from L. J. DeGroot) (57), and pSGhRXRα, encoding human retinoid X receptor α (hRXRα) (a gift from P. Chambon) (46), were used for synthesis of recombinant proteins by cell-free transcription-translation and in transient transfection experiments.

Plasmid pSV1773 contains a pseudo-wild-type (WT) SV40 genome with a frameshift mutation in the VP1-coding region (29); it was used as WT SV40 in transfection experiments to ensure that virion production and, consequently, second cycles of infection did not occur during single cycle growth curve experiments. The pseudo-WT SV40, pXS13, and the IBP-s binding site mutant variants of it, *pm*322C and *pm*322C×LS26, used in the cell-free transcription reactions have been described previously (77). Plasmid $(TRE+1)_2SV40L^*$ was constructed by insertion of two tandem copies in the sense orientation of the synthetic double-stranded oligonucleotide 5′-GAGGTTATTTCAGGCCATGG T-3′, corresponding to the WT SV40 +1 site TRE, at the *Pvu*II site (SV40 nucleotide [nt] 272) of mutant *pm*322C×LS26. Plasmid $mut(TRE+1)_2SV40L^*$ was similarly constructed by the insertion of two copies of a synthetic double-stranded oligonucleotide containing the mutant SV40+1 site TRE sequence 5′-GAGcTTATTTCAGcCCATGGT-3′ (the lowercase letters indicate the altered bases).

The plasmids used in the DNA bending experiment (see Fig. 8) were created by cloning the WT and mutant SV40+1 site oligonucleotides (see Fig. 2) into the *Sal*I site of the plasmid pBend2 (39) after this site had been filled in with the Klenow fragment of DNA polymerase. The DNA fragments were prepared from these plasmids by digestion with the indicated restriction endonucleases and gel purification. They are identical in length and sequence but differ in the position of the +1 site TRE relative to the ends of the DNA.

**Gel mobility shift assays (GMSAs).** Recombinant receptor proteins were synthesized in a coupled transcription-translation rabbit reticulocyte lysate system (Promega). The relative efficiencies of the syntheses were determined by incorporation of [$^{35}$S]methionine followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Similar amounts of $^{35}$S-labeled receptors in 1 to 5 μl of reticulocyte lysate were used in GMSAs performed as described previously (16) with minor modifications. Briefly, the binding reaction mixtures (16 μl) consisted of 10 mM HEPES (pH 7.5), 100 mM KCl, 1 mM dithiothreitol, 0.05 mM EDTA, 2.5 mM $MgCl_2$, 6% (vol/vol) glycerol, 2% (vol/vol) Ficoll, 4 μg of poly(dI-dC) · poly(dI-dC), and the indicated amount of in vitro-synthesized recombinant hTRα1 and/or hRXRα. After incubation at 4°C for 10 min, the indicated 5′ end-labeled, double-stranded oligonucleotide (0.2 to 5 ng; $2 \times 10^4$ cpm) was added and incubation was continued for 15 min at 4°C, followed by 15 min at room temperature. The DNA-protein complexes were resolved by electrophoresis at room temperature in native 4% polyacrylamide gels with 0.5× Tris-borate-EDTA as running buffer.

**Transient transfection assays.** CV-1P cells were grown at 37°C in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum. Transfections were performed by the DEAE-dextran method followed by chloroquine treatment as described previously (29). Eighteen hours before transfection, the freshly plated cells were placed in medium containing 3% charcoal-stripped fetal bovine serum (to deplete $T_3$); they remained in this medium throughout the experiment. The SV40 viral sequences present in the plasmids were excised from the cloning vectors and ligated to form monomer circles before transfection. The relative amounts of SV40 early and late RNAs present at the indicated times posttransfection were determined by quantitative S1 nuclease mapping as described previously (33) using the 5′-end-labeled probes indicated in Fig. 1B. The isolation of replicated viral DNA and its quantitative analysis by Southern blotting were performed as described previously (32). The relative amounts of SV40 major late RNA were calculated by normalization to both the relative amounts of SV40 early RNA and the relative amounts of replicated SV40 DNA present in the same samples.

**Cell-free transcription assays.** Cell-free transcription reactions were performed as described previously (77, 85). Final reaction volumes (25 μl) contained ~100 μg of HeLa cell nuclear extract (10 mg of protein/ml) and 200 ng of circular, plasmid SV40 DNA. The recombinant hTRα1, human hepatic nuclear factor 4 (hHNF4), and hRXRα proteins used in the experiments shown in Fig. 5 to 7 were synthesized in HeLa cells infected with recombinant vaccinia viruses containing the genes for these proteins (10, 60). Extracts prepared from cells infected in parallel with WT vaccinia virus served as a control. Appropriate amounts of these extracts were added to the transcription reactions as indicated either before or after incubation of the DNA templates with uninfected HeLa cell nuclear extract, followed by the addition of ribonucleoside triphosphates to 600 μM and Sarkosyl to 0.03%. Incubation was then continued at 26°C for 30 min (see Fig. 6C). The resulting SV40 early and late RNAs were analyzed by primer extension as described previously (77) with primers corresponding to SV40 nt 446 to 422 and 5178 to 5201, respectively.

**DNA bending assay.** DNA bending induced by the binding of TR/RXR heterodimers was analyzed with circularly permuted probes as described previously (79). The probes (Fig. 8A) were end labeled with either polynucleotide kinase or the Klenow fragment of DNA polymerase. After incubation of the DNAs for 15 min at room temperature with recombinant hTRα1/hRXRα from a cell-free translation system, the DNA-protein complexes were resolved in native 5% polyacrylamide gels (49) by electrophoresis at a field strength of ~12 V/cm for 4 h at 4°C.

## RESULTS

**TR/RXR heterodimers stably bind the initiation site of the SV40-MLP.** Previously, we noted that the two hormone receptor binding sites in the SV40-MLP, the +1 and +55 sites, contain direct repeats of the consensus half-site sequence, 5′-

| Oligo. | Sequence |
|---|---|
| | +1 |
| WT+1 | 5'-TCAGAGGTTATTTCAGGCCATGGT-3' |
| | 3'-AGTCTCCAATAAAGTCCGGTACCA-5' |
| -1C(half) | 5'-TTCAGAGGTc ATTTCA-3' |
| | 3'-AAGTCTCCAg TAAAGT-5' |
| -3C(half) | 5'-TTCAGAGc TTATTTCA-3' |
| | 3'-AAGTCTCg AGTAAAGT-5' |
| Mut+1 | 5'-TCAGAGc TTATTTCAGc CCATGGT-3' |
| | 3'-AGTCTCg AATAAAGTCg GGTACCA-5' |
| | +55 |
| WT+55 | GTTAAGGTTCGTAGGTCATGGA |
| | CAATTCCAAGCATCCAGTACCT |

FIG. 2. Sequences of the double-stranded oligonucleotides used in the GMSAs and cell-free transcription assays. Nucleotide numbers are relative to the initiation site of the SV40-MLP. Lowercase letters indicate alterations from the WT SV40 sequence. The underlines represent the locations of putative half-site sequences for binding of nuclear hormone receptors.

AGGTCA-3′, spaced by 4 bp (DR4) and 2 bp (DR2), respectively (77) (see Fig. 2). DR4s can frequently be bound by heterodimers of TR and RXR and function as TREs (49, 51, 52). To determine whether hTRα1, either alone or in combination with RXRα, binds this putative DR4 present in the SV40-MLP, we performed GMSAs using the recombinant proteins hTRα1 and hRXRα and an end-labeled oligonucleotide corresponding to the +1 site sequence (WT+1 oligonucleotide in Fig. 2) as probe. As shown in Fig. 3A, little or no DNA-receptor complex was observed in binding reactions containing either hTRα1 (lane 1) or hRXRα (lane 2) alone; however, DNA-receptor complexes efficiently formed on the WT+1 probe when both hTRα1 and hRXRα were present together in the same reaction (lane 3). These heterodimeric complexes were essentially eliminated by the addition of a 50-fold molar excess of unlabeled WT+1 oligonucleotide as competitor (lane 4). On the other hand, the addition of similar amounts of oligonucleotide Mut+1 (lane 5), containing a mutation in the +1 site TRE, or WT+55 (lane 6), containing a DR2 hormone response element (HRE), had no effect on the amount of protein-DNA complex formed. Thus, TR binds sequence specifically to the initiation site of the SV40-MLP largely as a heterodimer with RXR.

To determine the effect of thyroid hormone, $T_3$, on the stability of these heterodimeric receptor-DNA complexes, the binding reactions were repeated in the presence of $T_3$. Addition of $10^{-7}$ M (Fig. 3B, lane 2 versus lane 1) or even $10^{-6}$ M (Fig. 3B, lane 3 versus lane 1) $T_3$ did not significantly affect the amount of TRα1/RXRα bound to the WT+1 site probe DNA. However, it did slightly increase the electrophoretic mobility of the DNA-receptor complex (lanes 2 and 3 versus lane 1). Taken together with the similar observations of others (e.g., reference 49), these findings show that the binding of $T_3$ to its receptor likely affects the conformation of the TRα1/RXRα heterodimer bound to this TRE, but not its association with the DNA. Also, since TR/TR homodimers formed on DR4 TREs dissociate in the presence of $T_3$ (for examples, see references 54, 61, and 82), these latter findings confirm our conclusion that the DNA-receptor complexes formed on this TRE consist largely of TRα1/RXRα heterodimers.

To further determine the stability of these receptor-DNA complexes, we also measured their rate of dissociation from this TRE by repeating these assays, but with the addition after complex formation of unlabeled, double-stranded WT+1 oligonucleotide as competitor to prevent the rebinding to the radiolabeled probe of any receptor that had dissociated (Fig. 3C). Interestingly, little receptor-DNA complex was lost during the incubation at room temperature for 30 min, even in the presence of $T_3$ (Fig. 3C, lane 13 versus lane 8). Thus, we conclude that TR/RXR heterodimers can quite stably bind the

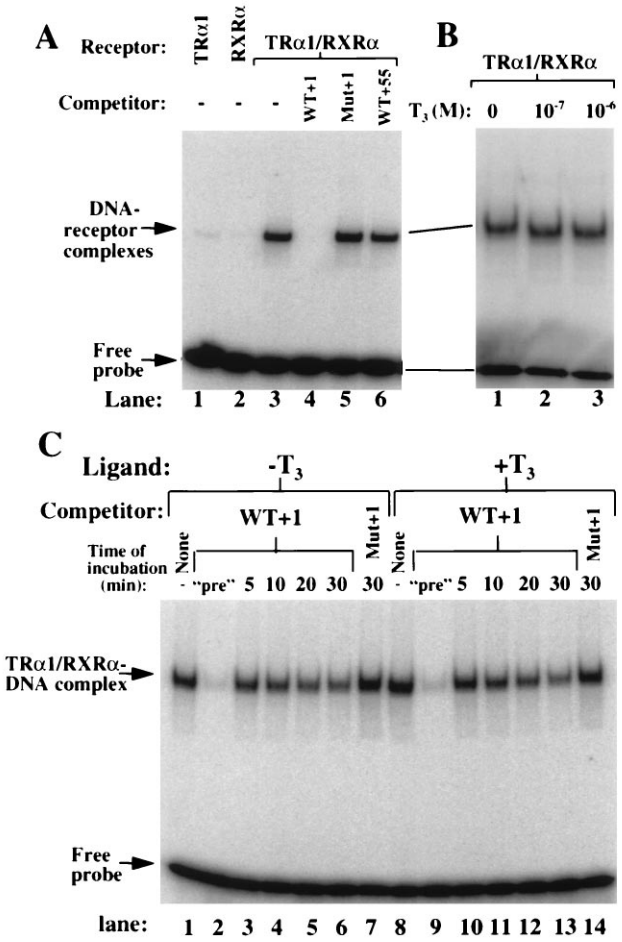


FIG. 3. Initiation site of the SV40-MLP is bound stably and sequence specifically by TR/RXR heterodimers. (A) Autoradiogram of GMSAs in which 5 ng of a probe consisting of radiolabeled, double-stranded, WT+1 oligonucleotide (sequence shown in Fig. 2) was incubated with 1 μl of recombinant hTRα1 (lane 1), hRXRα (lane 2), or hTRα1 plus hRXRα (lanes 3 to 6) synthesized in a reticulocyte lysate translation system. A 50-fold molar excess of unlabeled WT+1, Mut+1, or WT+55 double-stranded oligonucleotide (sequences shown in Fig. 2) was included as competitor in lanes 4 to 6, respectively. (B) Autoradiogram of GMSAs showing the effects of the thyroid hormone $T_3$ on the binding of TRα1/RXRα to this SV40 +1 site TRE. The radiolabeled WT+1 site probe was incubated with recombinant hTRα1/hRXRα as described for lane 3 of panel A, except that lanes 2 and 3 included as well $10^{-7}$ and $10^{-6}$ M $T_3$, respectively. (C) Autoradiogram of GMSAs showing that TRα1/RXRα stably associates with the SV40 +1 site TRE, even in the presence of $T_3$. Radiolabeled, double-stranded, WT+1 oligonucleotide probe was preincubated for 10 min at 4°C with recombinant TRα1 (1 μl) and RXRα (0.5 μl) synthesized in a reticulocyte translation system and then for 15 min at room temperature in the absence (lanes 1 to 7) or presence (lanes 8 to 14) of $10^{-7}$ M $T_3$. Afterward, a 50-fold molar excess of the indicated double-stranded oligonucleotide (sequences shown in Fig. 2) was added as competitor DNA and incubation was continued at room temperature for the indicated times. The samples were then stored on ice until loaded on the gel. In lanes 2 and 9, the unlabeled competitor DNA was added concurrently with the radiolabeled probe at the beginning of the preincubation period.

initiation site of the SV40-MLP and do so even in the presence of their cognate hormonal ligand.

**Transcription from the SV40 late promoter can be regulated by TR/RXR in a hormone-dependent manner in vivo.** Knowing that the SV40-MLP contains a potential TRE, we next determined whether transcription from this promoter can be modulated by $T_3$ and its receptor in vivo. Fortunately, CV-1 cells, a natural host for SV40, are deficient in thyroid receptors, yet contain RXRs (46). Thus, we could examine the effects of TR/RXR by overexpressing TRα1 in the cells. We accomplished this by cotransfecting CV-1 cells with WT SV40 DNA and pCDMα1, a plasmid encoding hTRα1. The cells were then incubated at 37°C for various lengths of time in the presence or absence of $10^{-7}$ M $T_3$. As a control, cells were cotransfected in parallel with WT SV40 DNA and pCDMα1's parental vector, pCDM8.

In the absence of $T_3$, overexpression of TRα1 in these cells resulted in a three- to fourfold decrease in the accumulation of both the major and minor SV40 late RNAs by intermediate times after transfection (Fig. 4A, lanes 4 and 8 versus lanes 2 and 6). However, by late times after transfection (i.e., 46 h), the effect of TR was largely abolished because the receptors were now limiting in amount (Fig. 4A, lane 12 versus lane 10; summarized in Fig. 4C) (77, 85). On the other hand, the accumulation of neither viral DNA (Fig. 4B) nor early RNA (Fig. 4A) was affected by the presence of TRα1. Thus, TR can specifically repress transcription from the SV40 late promoter, thereby resulting in a delay in the early-to-late switch in expression of the SV40 genome.

In the presence of $T_3$, this TRα1-mediated repression of the SV40-MLP was almost completely eliminated (Fig. 4A, lanes 5, 9, and 13 versus lanes 4, 8, and 12; summarized in Fig. 4C). The presence of $T_3$ also resulted in a small increase in the accumulation of SV40 late RNA even in the absence of exogenous TRα1 (Fig. 4A, lanes 3, 7, and 11 versus lanes 2, 6, and 10). This increase might have been caused by the presence of a small amount of functional TR in CV-1 cells or an indirect effect of $T_3$. Thus, we conclude that TR, presumably as heterodimers with the endogenous RXRs present in CV-1 cells, can specifically repress transcription from the SV40 late promoter, with this repression being largely reversible by the addition of $T_3$.

**Efficient repression of the SV40 late promoter by TR/RXR heterodimers in vitro.** To further confirm that this observed repression of the SV40 late promoter is the result of the binding of TR/RXR heterodimers, cell-free transcription assays were performed, assays in which the SV40 DNA template was preincubated with recombinant hTRα1 and hRXRα prior to the addition of the HeLa cell nuclear extract (Fig. 5). Afterward, the reaction mixtures were incubated at 26°C for 30 min to allow preICs to form, Sarkosyl was added to 0.03%, and, 30 s later, the ribonucleoside triphosphates were added (see also Fig. 6C). These conditions ensured that only a single round of transcription occurred (31).

When the template was preincubated with both hTRα1 and hRXRα, synthesis of the SV40 major and minor late RNAs was only 1/20th of the level observed in the absence of the receptors (Fig. 5, lane 4 versus lane 2). On the other hand, when the template was preincubated with hTRα1 alone, late RNA synthesis was reduced by only two-thirds (Fig. 5, lane 5 versus lane 2). No repression was observed with either hRXRα alone (Fig. 5, lane 6) or a different member of this receptor superfamily, hHNF4 (Fig. 5, lane 3). The abilities of these receptors to repress transcription from this promoter correlate well with their abilities to bind to the +1 site TRE (Fig. 3). Thus, we conclude that TRα1 as a heterodimer with RXRα

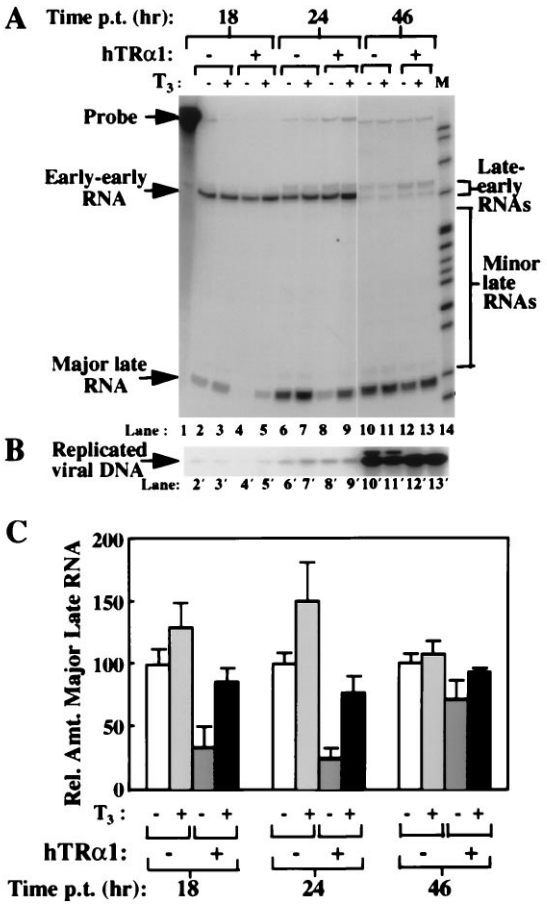


FIG. 4. Effects of hTRα1 and the thyroid hormone $T_3$ on the early-to-late switch in gene expression of SV40 during the lytic cycle of infection of CV-1P cells. (A) Autoradiogram of quantitative S1 nuclease mapping experiment used to determine the relative amounts of whole-cell SV40 early- and late-strand RNA accumulated by the indicated times posttransfection (p.t.). CV-1P cells were cotransfected with SV40 DNA (0.5 μg per 60-mm dish) excised from pSV1773 and 0.25 μg of the hTRα1-encoding plasmid pCDMα1 (lanes 4, 5, 8, 9, 13, and 14). As a control, cells were cotransfected in parallel with SV40 DNA and the parental vector of pCDMα1, pCDM8 (lanes 2, 3, 6, 7, 10, and 11). After transfection, the cells were incubated at 37°C in the absence (lanes 2, 4, 6, 8, 10, and 12) or presence (lanes 3, 5, 7, 9, 11, and 13) of $10^{-7}$ M $T_3$ for the times indicated, with replenishment of $T_3$ after 24 h. The hybridization reactions, performed with the probes indicated in Fig. 1B, contained 1/4, 1/8, and 1/80 of the RNA samples harvested at 18, 24, and 46 h, respectively. Lane M contained *Msp*I-cut pBR322 DNA as size markers. (B) Southern blot analysis of the replicated SV40 DNA present at the indicated times posttransfection. Three (lanes 2′ to 5′) and one (lanes 6′ to 13′) percent of the nucleic acid from the samples harvested in the experiment described for panel A were digested with *Dpn*I and *Eco*RI, separated by agarose gel electrophoresis, and probed with radiolabeled SV40 DNA. (C) Summary of the data obtained from three sets of experiments similar to the one shown in panels A and B. Plotted here are the amounts of the SV40 major late RNA accumulated in the cells relative to the amount accumulated in the absence of exogenous receptor and $T_3$ at the same time posttransfection, with internal normalizations performed as described in Materials and Methods. Error bars indicate standard errors of the mean.

can play a major role in repression of the SV40 late promoter. The synthesis of SV40 early RNAs from the same DNA template in the same transcription reactions was not affected by preincubation with these receptors (Fig. 5, lanes 3 to 6 versus lane 2). Thus, the repression observed here was promoter specific and not a trivial consequence of the sequestration of GTFs (i.e., squelching).

**TR/RXR represses transcription from the SV40 late promoter by preventing formation of preICs.** To begin to examine

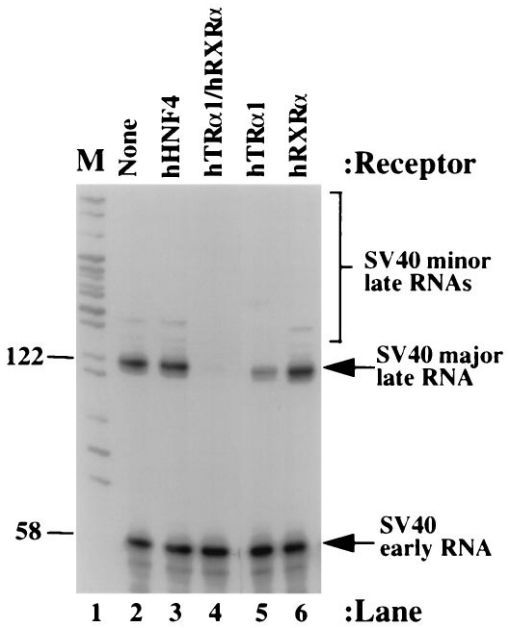


FIG. 5. Efficient repression of the SV40-MLP requires both TR and RXR. Shown here is an autoradiogram of a primer extension analysis of the SV40 early and late RNAs synthesized in a cell-free transcription system. The circular SV40 DNA template was incubated with the indicated recombinant receptor proteins, obtained from extracts of vaccinia virus-infected cells, prior to the formation of preICs as described in the legend to Fig. 6C. Lane 1, size markers consisting of *Msp*I-cut pBR322 DNA; lane 2, extract from WT vaccinia virus-infected cells (0.5 μl) added as a control; lane 3, hHNF4 (0.5 μl) added as a control, non-TRE-binding receptor protein; lane 4, hTRα1 (0.25 μl) plus hRXRα (0.25 μl); lane 5, hTRα1 (0.5 μl); lane 6, hRXRα (0.5 μl). The numbers on the left indicate sizes in nucleotides.

the mechanism by which TR/RXR heterodimers repress transcription from the SV40-MLP, we performed cell-free transcription assays as described above, but with recombinant hTRα1 and hRXRα added to the reaction mixtures either before or after addition of the HeLa cell nuclear extract (Fig. 6). As expected, addition of the recombinant hTRα1 and hRXRα prior to formation of the preICs resulted in sequence-specific repression of the WT SV40 late, but not early, promoter (Fig. 6A, lanes 3 and 4 versus lanes 1 and 2). However, when TRα1 and RXRα were added after formation of the preICs, late RNA synthesis was not affected (Fig. 6A, lanes 5 and 6 versus lanes 1 and 2). Thus, we conclude that the presence of the TRα1/RXRα heterodimers solely blocks the formation of preICs, rather than interferes with subsequent steps in transcription such as promoter clearance or elongation.

To verify that this observed repression of the SV40 late promoter by TRα1/RXRα was truly sequence specific, we also performed these assays with a mutant of SV40 containing a G→C change within the +1 site TRE at SV40 nt 322 (−3 relative to the initiation site of the MLP) as template. This mutation inhibits binding of receptors to this HRE (77). As expected, the TRα1/RXRα heterodimers failed to repress transcription from this mutant promoter even when they were added prior to formation of the preICs (Fig. 6, lane 10 versus lane 9). Preincubation of the extract with double-stranded competitor oligonucleotide containing the optimal +1 half-site sequence [−1C(half) oligo in Fig. 2] resulted in complete relief of repression by the TRα1/RXRα heterodimers (lane 8). On the other hand, preincubation with the same amount of oligonucleotide containing a mutated +1 half-site sequence [−3C (half) oligo. in Fig. 2] did not lead to relief of repression (lane 7). As expected, the synthesis of SV40 early RNA from these same templates in these same reactions was not affected by either these receptors or the competitor oligonucleotides. Thus, we conclude that the sequence-specific binding of TR/RXR heterodimers to the +1 site TRE represses transcription from the SV40 late promoter by preventing the formation of preICs.

The effect of the presence of thyroid hormone in this transcription system was examined by the inclusion of $10^{-7}$ M $T_3$ in the reactions analyzed in lanes 2, 4, and 6. Unfortunately, RNA synthesis was identical to that observed in the comparable reactions performed in the absence of $T_3$ (lanes 1, 3, and 5, respectively). Possible reasons for the failure of this cell-free system to mimic in vivo derepression or activation by $T_3$ are considered in the Discussion.

**Repression of transcription from the SV40-MLP by TR/RXR is position dependent.** Hormone response elements are usually situated upstream or downstream of the basal sequence elements of a promoter (reviewed in reference 8). Our finding that the TRE in the SV40-MLP overlaps the transcription initiation site is novel. Thus, we were interested in determining whether the ability of TR/RXR heterodimers to prevent the formation of the preICs on this promoter was a consequence of the unusual location of the TRE.

To examine the effect of the location of this TRE sequence on transcription from the SV40-MLP, we constructed a variant of SV40, $(TRE+1)_2SV40L^*$, in which two copies of this +1

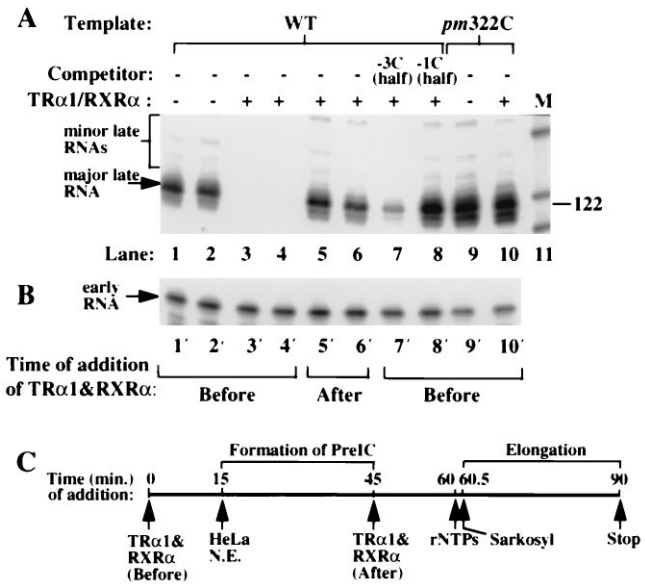


FIG. 6. Binding of TR/RXR heterodimers to the initiation site of the SV40-MLP prevents the formation of functional preICs in vitro. (A and B) Autoradiograms showing the effects of the order of addition of TR/RXR and HeLa cell nuclear extract on cell-free transcription from the SV40 late (A) versus early (B) promoters. The order-of-addition experiments were performed essentially as described previously (31) following the protocols indicated schematically in panel C. The WT and TRE mutant circular SV40 DNA templates were incubated with HeLa cell nuclear extract at 26°C. Recombinant hTRα1 (0.25 μl) and hRXRα (0.25 μl), obtained from extracts of HeLa cells infected with recombinant vaccinia viruses, were incubated with the templates either before (lanes 1 to 4 and 7 to 10) or after (lanes 5 and 6) addition of the HeLa cell nuclear extract. In lanes 1, 2, and 9, extract from HeLa cells infected with WT vaccinia virus was added as a control. In lanes 7 and 8, the reactions also included a 100-fold molar excess of the indicated double-stranded oligonucleotide (sequences shown in Fig. 2) added as competitor at the same time as TRα1/RXRα. Shown here is a primer extension analysis of the viral RNAs synthesized in each reaction. M, size markers of *Msp*I-cut pBR322 DNA. (C) Schematic diagram indicating the order in which components were added to the transcription reactions analyzed in the experiments shown in Fig. 5 and panels A and B of this figure.

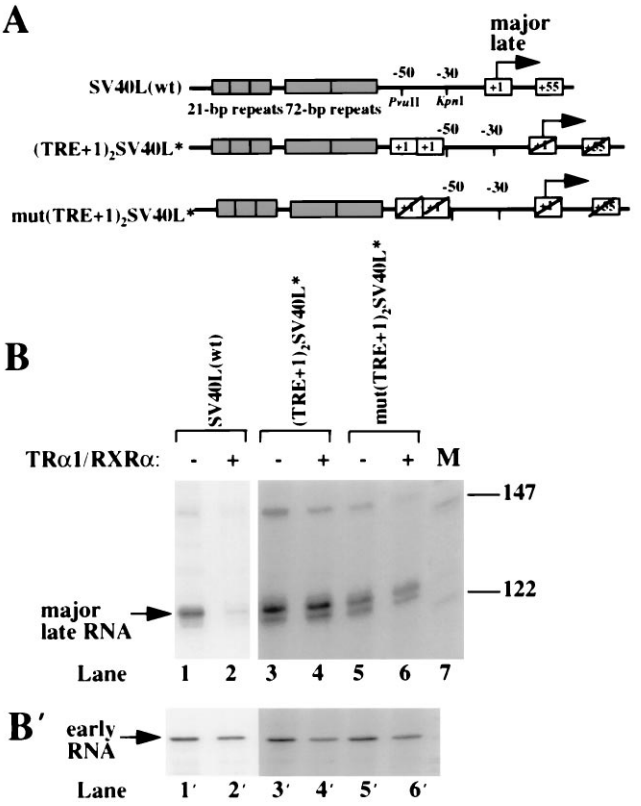


FIG. 7. Effect of location within the SV40 late promoter of the +1 site TRE on repression by TR/RXR heterodimers in vitro. (A) Schematic diagrams of the late promoter regions of the SV40 DNAs used as templates in the experiments shown in panels B and B′. SV40L(wt) is WT SV40 DNA. The two mutants differ from WT SV40 by (i) the insertion at −53 relative to the major late initiation site of two tandem copies of the +1 site TRE (indicated by the boxes labeled "+1") and (ii) mutations inactivating binding of receptors to some of these HREs (indicated by diagonal lines) (77). (B and B′) Cell-free transcription experiments showing that relocation of the +1 site TRE to a position upstream of the basal elements of the SV40-MLP eliminates transcriptional repression by hTRα1/RXRα. The assays were performed as described for Fig. 6, lanes 1 to 4, except for the omission of sarcosyl. Shown here is a primer extension analysis of the viral RNAs synthesized in each reaction. The exposure time for lanes 1 and 2 was one-half of the exposure time for lanes 3 to 7.

site TRE sequence were inserted in tandem 55 bp upstream of the transcription initiation site of the SV40-MLP (Fig. 7A). This plasmid also contains mutations at the +1 and +55 site HREs to inactivate the two major native HREs. As a control, we constructed an additional variant, mut(TRE+1)$_2$SV40L*, in which the +1 site TRE inserted in tandem at −55 had been mutationally inactivated as well (Fig. 7A).

Cell-free transcription assays were performed with these SV40 DNA variants as templates (Fig. 7B). As expected, the addition of TRα1/RXRα heterodimers did not significantly repress transcription from the late promoter of the variant in which the inserted TREs had been mutated (lane 6 versus lane 5). Surprisingly, neither did they repress transcription of either the major or minor late RNAs synthesized from the variant containing two WT copies of the +1 site TRE inserted 55 bp upstream of the major late initiation site (lane 4 versus lane 3). Thus, we conclude that repression by TR/RXR heterodimers in the context of the SV40 late promoter is dependent upon the location of the TRE. This finding suggests that specific, stereo-restrained protein-protein interactions and/or competition between receptors and components of the general transcriptional machinery for binding to the template likely is responsible for the observed repression.

**The +1 site TRE of the SV40-MLP is bent by the binding of TR/RXR heterodimers.** The binding of TR to some TRE sequences induces bending of the DNA (40, 47, 49). To test whether TRα1/RXRα heterodimers induce DNA bending when bound to the SV40 +1 site TRE, we performed GMSAs using a set of radiolabeled probes that were identical in size and sequence but were different in the position of the WT+1 site TRE relative to the ends of the DNA (Fig. 8A). Each reaction contained the same amount of recombinant TRα1 and RXRα (Fig. 8B).

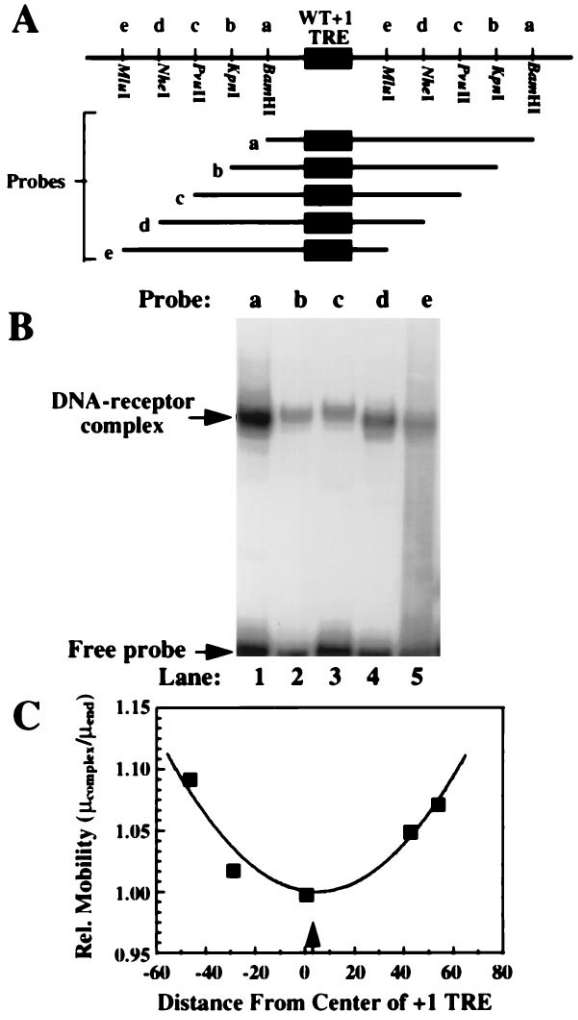


FIG. 8. Binding of TR/RXR heterodimer to the SV40 +1 site TRE induces bending in the DNA. (A) Schematic representation of the circularly permuted TRE-containing DNA probes used in the experiment shown in panel B. Plasmid pBend2(WT+1 TRE) was digested with *Bam*HI, *Kpn*I, *Pvu*II, *Nhe*I, or *Mlu*I to generate the 144-bp DNA fragments labeled a, b, c, d, and e, respectively. The solid boxes represent the inserted SV40 +1 site TRE. (B) Autoradiogram of GMSAs showing the differential mobilities of the DNA-protein complexes formed on the circularly permuted probes indicated in panel A. Each of the radiolabeled probes was incubated with reticulocyte lysate-synthesized hTRα1 (1.5 μl) plus RXRα (1 μl) (lanes 1 to 5). (C) Plot of the mobilities ($\mu_{complex}/\mu_{end}$) of the hTRα1/hRXRα-containing complexes as a function of the position of the center of the TRE relative to the ends of the probes, with normalization to the mobility of probe c. The curve connecting the points is a calculated best-fit cosine function for these data. The arrow, located at the position of maximum relative mobility on the fitted curve, indicates the location of the center of the bend in the DNA.

Protein-DNA complexes formed on the probes in which the binding site was near the middle of the DNA migrated more slowly (lanes 2, 3, and 4) than protein-DNA complexes formed on probes in which the binding site was near either end (lanes 1 and 5). This finding indicated, as expected, that the bend in the DNA induced by binding of the TRα1/RXRα heterodimers occurs within the TRE (summarized in Fig. 8C). No detectable differences in mobility were observed among the unbound probes (Fig. 8B, free probe) (84). In addition, no distinct complexes were formed on probes containing a mutated +1 site TRE (data not shown and reference 84). Thus, the differential migration of the bound, circularly permutated DNA fragments was the result of TRα1/RXRα heterodimer-induced DNA bending. Furthermore, the addition of $T_3$ had little, if any, effect on the observed DNA bending (data not shown and reference 84). Thus, the $T_3$-mediated derepression of the SV40 late promoter observed in vivo is probably a consequence neither of release from the DNA template nor of significant alteration in the bending of the DNA near the +1 site TRE.

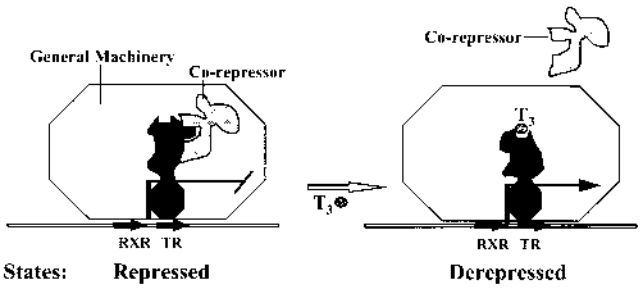


FIG. 9. Model for repression and derepression of the SV40-MLP by TR/RXR and the thyroid hormone $T_3$, respectively. In the absence of $T_3$, the TR/RXR heterodimer, in association with a corepressor, is bound to the +1 site TRE present in the SV40-MLP, leading to inactivation of the basal transcriptional machinery via multiple inhibitory protein-protein interactions and, possibly, alterations in the structure of the DNA template. In the presence of TR's natural ligand, $T_3$, a conformational change in TR is induced, thereby reversing the repression of basal transcriptional activity by causing removal of the corepressor and inhibitory protein-protein interactions and enabling exposure of TR's activation domain and formation of active preICs. LBD, ligand binding domain; DBD, DNA binding domain.

## DISCUSSION

We identified here a functional TRE overlapping the initiation site of the SV40-MLP (Fig. 1 to 5). Using recombinant hTRα1 and hRXRα, we showed that the initiation site of the SV40-MLP is very stably bound by TRα1/RXRα heterodimers in both the absence and the presence of TR's natural ligand, $T_3$ (Fig. 3). We went on to show that, in the absence of $T_3$, TRα1/RXRα heterodimers specifically repress transcription from the SV40 late promoter both in vivo (Fig. 4) and in vitro (Fig. 5 and 6). On the other hand, TRα1 by itself binds only weakly as homodimers or monomers to this TRE (Fig. 3); thus, as expected, TRα1 represses transcription from this promoter only weakly (Fig. 5). We further showed that repression by TRα1/RXR is largely relieved in vivo by the presence of $T_3$ (Fig. 4). Therefore, we conclude that the SV40 late promoter does, indeed, contain a functional TR/RXR binding site that enables hormonal regulation of expression of the late genes of SV40 in cells containing these receptors.

Experiments aimed at understanding the mechanism of repression indicated that (i) the binding of TRα1/RXRα to the SV40 +1 site TRE prevents the formation of functional preICs at the upstream, minor late promoters as well as at the MLP (Fig. 6) and (ii) the precise location of this TRE within the SV40 late promoter is critical for its activity (Fig. 7). Since the TRα1/RXRα heterodimers remain stably bound to this TRE in the presence of $T_3$ (Fig. 3), we conclude that the mechanisms of repression and derepression of basal transcription from these multiple SV40 late promoters by these receptors and their ligands, respectively, probably involve active processes in which changes occur in crucial, stereospecified protein-protein and protein-DNA interactions necessary for the formation of functional preICs (Fig. 9).

**Mechanism of repression of the SV40 late promoters by TR/RXR.** We demonstrated here that unliganded TR/RXR heterodimers repress transcription from the SV40 late promoters in a context-dependent manner by preventing the formation of functional preICs but do not inhibit their activity once preICs have been allowed to properly form (Fig. 5 to 7). Similar observations have recently been reported for unliganded TR homodimers bound to synthetic, palindromic TREs (22, 23, 71). Thus, the presence of these receptors probably interferes with an early stage in the proper formation of preICs. The precise mechanism(s) by which the context-specific binding of TR/TR or TR/RXR dimers to some promoters prevents the formation of functional preICs remains to be determined. It was previously shown that the repression domain of TR can interact with the basal transcriptional factors TFIIB (2, 3, 22) and, possibly, TBP (23). These receptor-GTF protein-protein interactions might preclude these basal transcription factors from properly associating with other general transcription factors. However, since other regions of TR which do not interact with these basal factors are also required for TR-mediated repression (3, 19), these interactions are not sufficient. Recent work from several groups (4, 11, 14, 35, 42, 66, 68) has revealed the presence of a new family of nuclear corepressor proteins, called SMRT and TRAC, which likely mediate repression by unliganded thyroid receptors by both interacting with TRs and containing a repressor domain which might interact with the basal transcriptional machinery or other yet-to-be identified cofactors. Preliminary data from the Mertz laboratory indicate that the cotransfection into CV-1P cells as described in Fig. 4 of a SMRT-encoding plasmid (a gift from R. M. Evans; see reference 14) along with pCDMα1 and SV40 DNA leads to increased repression of transcription from the SV40 late promoter (57a). This latter finding suggests that TR/RXR heterodimers may repress transcription from the SV40 late promoter in a corepressor-dependent manner.

TR-induced bending of the promoter DNA has also been hypothesized as a mechanism for transcriptional regulation of thyroid hormone-responsive genes by TR (49). Although we observed bending of the SV40 +1 site TRE upon binding of TRα1/RXRα (Fig. 8), this bending was not affected significantly by the presence of $T_3$ (data not shown and reference 84). Thus, the minor change we observed in the mobility of this receptor-DNA complex upon the addition of $T_3$ (Fig. 3B) is likely the result of a change in the conformation of the thyroid receptor, not the DNA. Therefore, although we cannot yet rule out this possibility, we consider it unlikely that repression and derepression of the SV40 late promoter by TR/RXR and $T_3$, respectively, are predominantly consequences of changes being induced in the conformation of the promoter DNA.

A third possibility for the mechanism of repression of the SV40 late promoter by TR/RXR is that TR/RXR occupation of the initiation site of the MLP excludes binding of RNA polymerase II, TFIID, or other GTFs to this region of the promoter. We cannot yet definitively exclude this possibility. However, we consider it unlikely for the following reasons.

First, while liganded TRα1/RXRα heterodimers can stably bind the +1 site TRE (Fig. 3B and C), they do not repress transcription from the SV40-MLP in vivo (Fig. 4). Thus, occupation of the initiation site, by itself, is not sufficient to inhibit access of the general transcription machinery to this promoter. Rather, these factors may be able to associate concurrently with the same or overlapping sequences of the promoter by interacting with different components of it—e.g., the major versus minor groove of the DNA helix. Second, transcription from the upstream, minor late promoters of SV40 was also repressed but only when the template was incubated with TRα1/RXRα prior to formation of the preICs (Fig. 6, lanes 3 and 4 versus lanes 5 and 6). Thus, binding of TR/RXR to the +1 site TRE around SV40 nt 325 inhibits the formation of functional preICs at SV40 nt 264 and 232 as well, sites far removed from this TRE. Third, we have also performed cotransfection and order-of-addition experiments similar to the ones shown in Fig. 4 and 6 with the SV40 mutant pm322C, a mutant defective in the +1 site HRE, and a highly purified preparation of IPB-s (which contains several members of the steroid/thyroid hormone receptor superfamily, some of which bind the +55 site of the SV40-MLP [77]): once again, the binding of receptors to the promoter repressed transcription from the SV40-MLP even though these receptors bound 50+ bp downstream of the site of initiation (77, 84) and did so by blocking the formation of functional preICs (84). Therefore, we conclude that the mechanism of repression of the late genes of SV40 by TR/RXR heterodimers is probably an active one that involves receptor-GTF and corepressor-GTF interactions (Fig. 9).

**Mechanism of derepression by $T_3$.** We also showed here that the presence of the thyroid hormone $T_3$ leads to relief of repression of the SV40 late promoter by TR/RXR in vivo (Fig. 4). Unfortunately, we have yet to mimic successfully this $T_3$-mediated derepression in our cell-free transcription system (Fig. 6) (data not shown and reference 84), possibly because our HeLa cell nuclear extract may lack sufficient quantities of necessary factors. For example, the exogenously added TR/RXR heterodimers may be in molar excess to the endogenous corepressors and coactivators. The precise mechanism of this depression remains unclear. Nevertheless, recent studies have shown that the binding of $T_3$ to TR results in (i) the dissociation from TR of TFIIB (3, 71), TBP (23), and corepressor proteins (14, 35, 42, 66); (ii) the association of TR with coactivator(s) (12, 30, 42); (iii) changes in the conformation of TR (e.g., reference 6); and (iv) the dissociation of TR/TR homodimers (for examples, see references 81 and 82). In the prior studies of Fondell et al. (22), the derepression of transcription they observed upon addition of ligand may have been a consequence of the disassociation of TR/TR homodimers. That possibility was excluded here by showing clearly that TR/RXR heterodimers were the primary form of the receptors responsible for both binding (Fig. 3) and repression (Fig. 5) on the SV40 +1 site TRE. Interestingly, we observed derepression of basal transcription, but not activation in vivo (Fig. 4) as has been reported by others with other TREs in other promoters (for examples, see references 18, 41, and 70). We speculate that derepression of the SV40 late promoter by $T_3$ may have been due to the dissociation of TFIIB, TBP, and/or corepressors from TR, thereby allowing for the proper assembly of functional preICs (Fig. 9); however, activation failed to occur because (i) coactivators may have been limiting in our assay system, in which the template and TR-encoding plasmids were replicating, and/or (ii) the location of the TRE directly at the site of initiation may be incompatible with enabling activation.

We have also found that the presence of SV40 large T antigen can partially substitute for $T_3$ to enable derepression of this TR-mediated repression of the SV40 late promoter (84). We speculate that T antigen may be able to substitute functionally for ligand in relieving repression. The mechanism of this derepression, presently under investigation, probably involves protein-protein interactions between T antigen and GTFs (17, 38), corepressors, coactivators, and receptors (57a).

**Physiological function of the +1 site TRE in regulating SV40 gene expression.** In nature, SV40 is a fairly ubiquitous virus found in a variety of primates including rhesus monkeys, African green monkeys, and humans (72). It is primarily isolated from kidney fibroblasts (69). The cell line CV-1P, used in the in vivo experiments reported here, is derived from African green monkey kidney cells (53). Interestingly, this particular cell line contains little functional TR (46). Thus, it was ideal for use in the model experimental system reported here. These facts lead one to question the physiological relevance of this TRE in the regulation of SV40 gene expression. However, both thyroid and retinoid X receptors are widely distributed among tissues in mammals, including kidney (50, 63). In addition, numerous other members of the steroid/thyroid hormone receptor superfamily present in a variety of tissues also bind putative HREs identified within the SV40 late promoter (77, 85). Thus, when SV40 infects whole animals, it is highly likely that its late promoter can be regulated by these receptors and their ligands. Therefore, it is likely that the presence of certain hormones can influence virus production in whole animals.

**Possible direct effects of hormones on other DNA viruses.** We showed here that a natural hormone can directly affect the expression of a DNA virus. Tomkins, Yamamoto, and colleagues first demonstrated more than two decades ago that the expression of another virus, murine mammary tumor virus, can be directly affected by another hormone, glucocorticoid (references 56, 59, and 62 and references cited therein). Given that HREs appear to exist in the promoters of many viruses, including some clinically relevant human ones such as human immunodeficiency virus type 1 (15, 45), human hepatitis B virus (24, 74, 83), adenovirus (77), and herpes simplex virus type 1 (58), we speculate that many viruses may be subject to regulation by hormones and their receptors. If true, it may be possible to develop a new class of antiviral drugs consisting of natural or synthetic ligands to the receptors which bind specific HREs present in crucial regulatory promoters of these viruses.

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