BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 280–284 (1997)
ARTICLE NO. RC977285

The Gene Regulating Activity of Thyroid Hormone Nuclear Receptors Is Modulated by Cell-Type Specific Factors

Kwang-huei Lin, Shen-liang Chen, Xu-Guang Zhu,* Hsing-ying Shieh, Peter McPhie,† and Sheue-yann Cheng*,[1]

*Graduate Institute of Clinical Medicine, Chang-Gung College of Medicine and Technology, Taoyuan, Taiwan; and †Laboratory of Biochemical Pharmacology, National Institute of Diabetes, Digestive, and Kidney Diseases and *Gene Regulation Section, Laboratory of Molecular Biology, DBS, National Cancer Institute, National Institutes of Health, Bldg. 37, Rm. 2D24, 37 Convent Drive MSC 4255, Bethesda, Maryland 20892-4255*

Received August 11, 1997

To understand whether the transcriptional activity of thyroid hormone nuclear receptors (TRs) is modulated by cell-type specific factors, full length TR subtype α1 (TRα1) and β1 (TRβ1) cDNAs were cloned from human hepatoma cell lines: HA22T, SK-Hep-1 and HepG2. The cloned receptor bound to the thyroid hormone 3,3′,5-triiodo-L-thyronine (T_3) and the thyroid hormone response elements (TREs) similarly to those cloned from other tissues. They exhibited T_3- and TRE-dependent transactivation activities, indicating these TRs were transcriptionally active. The lipogenic malic enzyme (ME), a T_3-target gene in liver, was stimulated ~3- and 1.5-fold by T_3 in HA22T and SK-Hep-1, respectively. The T_3-stimulated ME gene expression was inhibited in HA22T, but stimulated in SK-Hep-1 cells by insulin. These results suggest that the gene regulating activity of TRs was modulated by cell-type specific factors. Furthermore, these cell-type specific factors could modulate the cross talk between TR- and insulin receptor-mediated pathways.

The thyroid hormone, 3,3′,5-triiodo-L-thyronine (T_3), promotes growth, induces differentiation and regulates metabolic functions. Most of these effects are mediated by the interaction of T_3 with thyroid hormone receptors (TRs). Two TR genes, TRα and TRβ, located on chromosome 17 and 3, respectively, yield three TR isoforms, TRα1, TRβ1 and TRβ2 (1, 2). TRα1 and TRβ1 are expressed in most of the tissues, whereas TRβ2 is primarily expressed in brain (1). TRs regulate the transcription of T_3 target genes by binding to specific DNA sequences, known as the thyroid hormone response elements (TREs). The TREs consist of consensus sequences of DNA with the half-site binding motifs as a palindrome (Pal), as a direct repeat separated by four nucleotides (DR+4) or as an inverted repeat separated by six nucleotides (F2). As in other members of the nuclear receptor superfamily, the sequence of the TR can be divided into three different domains: the amino-terminal region (domain A/B), the central region (domains C+D), and the carboxyl-terminal region (domain E). The central region contains two zinc fingers and is involved in binding of receptors to TRE. Domain E is highly complex, in addition to its function of hormone binding, it contains sequences which mediate dimerization and transactivation (2).

In spite of recent progress, the mechanism of thyroid hormone action is not entirely clear. One critical issue is whether the diverse action of TRs is mediated by cell type-dependent factors. One strategy to address this critical issue is to use established cell lines, derived from a T_3 target tissue, which exhibit different functional characteristics. Cell lines, HA22T, SK-Hep-1 and HepG2 which were derived from human hepatocellular carcinoma (HCC), provided such a possibility. HA22T and SK-Hep-1 are poorly differentiated and their growth is stimulated by T_3, whereas HepG2 is well differentiated and its growth is less sensitive to T_3 (3). In the present study, we cloned TRα1 and TRβ1 from these three cell lines and characterized their T_3, TRE binding and transactivation activities. We found that the TRs from these three cell lines were transcriptionally active. However, the extent of T_3-stimulated expression of the important lipogenic malic enzyme gene (ME), a T_3-target gene in liver, not only varied with

[1] To whom correspondence should be addressed. Fax: (301) 480-9676.

Abbreviations: T_3, 3,3′,5-triiodo-L-thyronine; TR, thyroid hormone receptor; TRβ1, human TR subtype β1; TRα1, human TR subtype α1; TRE, thyroid hormone response element; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; F2, chicken lysozyme TRE; DR4, direct repeat of the half-site binding motifs separated by 4 nucleotides; Pal, palindromic TRE; ME, the rat malic enzyme gene.

0006-291X/97 $25.00

cell type, but also exhibited opposite sensitivity toward insulin, suggesting that the gene regulating activity of TRs was modulated by cell-type specific factors.

MATERIAL AND METHODS

Cloning of TRs from hepatocellular carcinoma cells. Total RNA was prepared from each cell line by guanidinium thiocyanate method. Reverse transcription of RNA followed by polymerase chain reaction (PCR) was carried out as described by Cook *et al.* (4). The cDNAs obtained were used as templates for PCR which was carried out in 10 mM Tris HCl/pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM dNTP, with the isoform-specific 5′ and 3′ primers , and Taq polymerase (0.5 μl; 5 units/μl). The amplified cDNAs were purified, and ligated onto pGEM-T vectors (Promega; Madison, WI). TR isoform containing plasmids were confirmed by restriction enzyme mapping and Southern blot analysis.

Determination of ME gene expression by reverse transcription and quantitative PCR (RT-QPCR). The expression of ME gene was determined by RT-QPCR as described by Sugimoto *et al.* (5) which utilized 2 sets of primers (ME, 100 ng and actin, 25 ng) in the same tube for simultaneous amplification of cDNAs by PCR. Conditions were defined in the preliminary experiment to ensure that the cDNAs amplified were in the linear range. The cDNAs were applied to a 5% polyacrylamide gel and the band intensities were determined by the phosphoimager (Fuji, BAS 2000).

Electrophoresis mobility shift assay (EMSA). ^{32}P-labeled F2 was prepared as described (6). TR proteins were synthesized by *in vitro* transcription/translation using TNT-coupled reticulocyte kit according to the manufacturer's instructions (Promega, Madison, WI). The synthesized TR proteins were quantified by measuring the intensity of the ^{35}S-labeled protein bands after SDS-PAGE. For EMSA, identical amounts of TRs were incubated with the ^{32}P-labeled F2 in the presence or absence of the retinoid X receptor. After electrophoresis, TR homodimers and heterodimers were visualized by autoradiograph as described by Meier *et al* (6).

Binding of [3′-^{125}I]T_3 to TRs. Five μl of the lysate containing the *in vitro* translated TR proteins or 30 μg of the nuclear extracts of HCC cells were incubated with 0.5 nM of [3′-^{125}I]T_3 in the presence of increasing concentrations of unlabeled T_3. The TR-bound [3′-^{125}I]T_3 was separated from the free as described by Lin *et al* (7).

Determination of the transcriptional activity of endogenous TRs. To determine the T_3-dependent transactivation activity of the endogenous TRs in HCC cells, the TRE-containing luciferase reporter genes were transfected into cells with or without T_3 by lipofectamine method according to the manufacturer's instructions (Gibco Inc.). After 48 hrs, the cells were harvested and 100 μg protein were assayed for luciferase activity (8).

RESULTS

TRα1 and TRβ1 from HA22T, SK-Hep-1 and HepG2 cells bind to T_3 with high affinity. To evaluate the T_3 binding activity of TRs from HA22T, SK-Hep-1 and HepG2 cells, cDNAs encoding TRα1 and TRβ1 were isolated from these cells and cloned into T7-expression vectors. For controls, TRα1 and TRβ1 were similarly isolated from normal human liver and placenta, respectively and cloned into T7-expression vectors. The identity of these clones was confirmed by restriction enzyme mapping and Southern blot analysis. TRα1 and TRβ1 proteins were synthesized from the T7-expression vectors by *in vitro* transcription/translation. Figure 1 shows that the sizes and the patterns of the synthesized TRα1 (lanes 1-3) and TRβ1 (lanes 6-8) from the three HCC cell lines were indistinguishable from those of the controls shown in lane 4 (TRα1 from normal human liver) for TRα1, lane 5 (TRβ1 from human placenta) and lane 9 (TRβ1 cloned by Weinberger *et al*; 9) for TRβ1, respectively. Intact *in vitro* translated TRα1 and TRβ1 had molecular weights of 49K and 55K, respectively, but lower molecular weight proteins, due to initiation at the downstream ATGs, were detected (9, 10). These results indicate that TRα1 and TRβ1 from HA22T, SK-Hep-1 and HepG2 cells had the same molecular weights as those cloned from other tissues (9, 10).

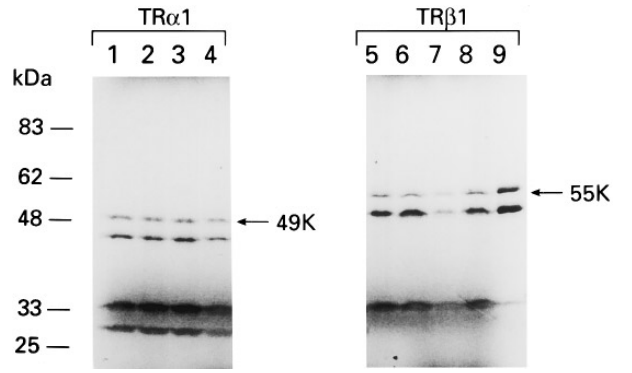

FIG. 1. The size of the *in vitro* translated TRα1 and TRβ1 cloned from HCC cells. cDNAs of TRα1 and TRβ1 were isolated from HCC cells and cloned into T7-expression vectors. TRα1 and TRβ1 were synthesized *in vitro* using [^{35}S]methionine and the receptor proteins (10 μl of lysate) were analyzed by SDS-PAGE. Lanes 1-4, TRα1 from SK-Hep-1, HA22T, HepG2 and normal liver, respectively. Lanes 5-9, TRβ1 from human placenta (cloned in the present study), SK-Hep-1, HA22T, HepG2 and human placenta cloned by Weinberger *et al.* (9), respectively.

Whether the TRs cloned from these HCC bound to T_3 was evaluated by competitive T_3 binding assay. Figure 2 shows that the binding of [^{125}I]T_3 to the *in vitro* translated TRβ1 from HA22T, SK-Hep-1 and HepG2 cells was competitively inhibited by the unlabeled T_3 in a concentration-dependent manner. The displacement curves for the TRβ1 from the three HCC cells were indistinguishable from that of TRβ1 cloned previously from placenta by Weinberger *et al* (9). Similar binding experiments were carried out to compare the binding of TRα1 from HCC cells with those from TRα1 isolated from normal human liver and skeletal muscle (10) (data not shown). Analysis of binding data indicate that the TRβ1 and α1 cloned from HCC cells bound to T_3 with Kds similar to those determined previously for TRs cloned from other tissues (Kds in the range of 0.3 - 0.7 nM; 9, 11), indicating that these TRs bound to T_3 with high affinity.

TRα1 and TRβ1 from HA22T, SK-Hep-1 and HepG2 cells bind to the thyroid hormone response elements.

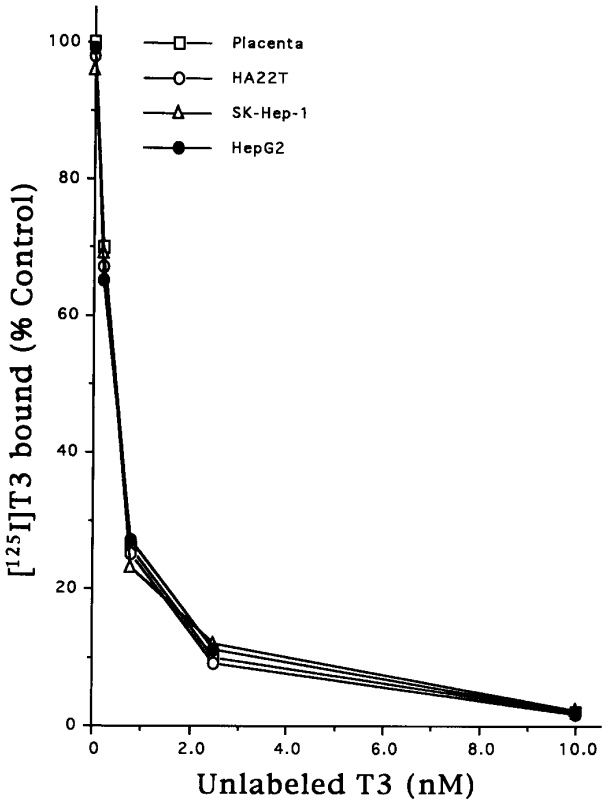

FIG. 2. Binding of TRβ1 from HCC cells to T_3. The *in vitro* translated TRβ1 from HCC cells (5 μl of lysate) as described in Fig. 1 was incubated with 0.5 nM of [^{125}I]T_3 in the absence or presence of increasing concentration of unlabeled T_3. The free and bound [^{125}I]T_3 were separated as described in Methods. HA22T, ○ ; SK-Hep-1, △; HepG2, ●; TRβ1 cloned from placenta, □.

To evaluate the DNA binding activity of the TRs cloned from these HCC cells, we used electrophoresis mobility shift assay (EMSA). Lanes 6, 8 and 10 of Fig. 3A show that TRβ1 from SK-Hep-1, HA22T and HepG2, respectively, bound to F2 mainly as a homodimer. Lanes 2 and 4 were the controls using TRβ1 cloned from placenta by Weinberger *et al* (9) and by us in the present studies, respectively. The binding of F2 to TRβ1 from these three cell lines was similar to those of the controls (lanes 6, 8 and 10 vs. lanes 2 and 4). TRs are known to heterodimerize with the retinoid X receptors (RXRs) on TREs (1). Therefore, we also evaluated the heterodimerization of TRs from HCC cells with RXRβ on F2. Lanes 7, 9 and 11 show that TRβ1 from these cell lines heterodimerize with RXRβ equally well as compared to the controls (lanes 3 and 5).

TRα1 bound to F2 differently from TRβ1 (12). As shown in lanes 4, 6 and 8, TRα1 from HCC cells bound to F2 both as a monomer and as a homodimer. In the presence of RXRβ, more intense heterodimer bands were seen (lanes 5, 7 and 9). No significant qualitative or quantitative differences between the binding of F2 to TRα1 from HCC cells and to the control (lanes 4, 6 and 8 vs. lane 2; lanes 5,7 and 9 vs. lane 3) were detected. Binding of TRα1 and TRβ1 from HCC cells to Pal or DR+4 was also evaluated and found to be similar to the TRs cloned from normal tissues (data not shown). These results indicate that TRs from HCC had functional DNA binding activity.

The transactivation activity of the endogenous TRs in HA22T, SK-Hep-1 and HepG2 cells are T_3- and TRE-dependent. To assess whether the endogenous TRs in HA22T, SK-Hep-1 and Hep-G2 cells were transcrip-

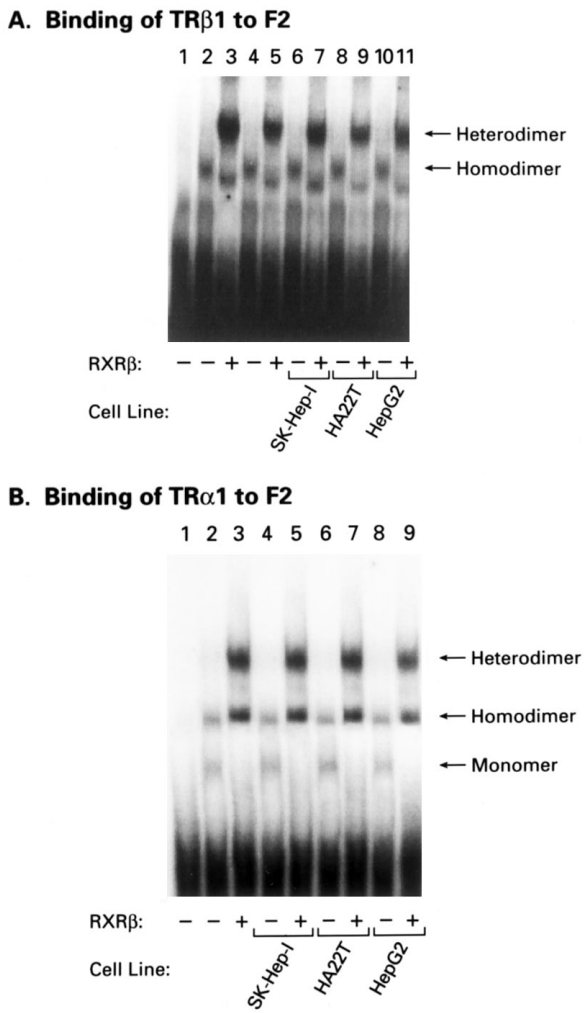

FIG. 3 Binding of TRα1 and TRβ1 to F2 by electrophoresis mobility shift assay. An equal amount of *in vitro* translated TRβ1 (A) or TRα1(B) was incubated with [^{32}P]F2 in the absence (lanes 2, 4, 6, 8 and 10) and presence of RXRβ (lanes 3, 5, 7, 9 and 11). The bound TRs were analyzed by gel electrophoresis as described in Methods. (A). lane 1, unprogrammed lysate as control; lanes 2 and 3, TRβ1 from human placenta cloned by Weinberger *et al.* (9) lanes 4 and 5, TRβ1 from human placenta cloned by us in the present studies; lanes 6 and 7, TRβ1 from SK-Hep-1; lanes 8 and 9, TRβ1 from HA22T; lanes 10 and 11, TRβ1 from HepG2. (B) Lane 1, unprogrammed lysate as control; lanes 2 and 3, TRα1 from human skeletal muscle cloned by Nakai *et al.* (10); lanes 4 and 5, TRα1 from SK-Hep-1; lanes 6 and 7, TRα1 from HA22T; lanes 8 and 9, TRα1 from HepG2.

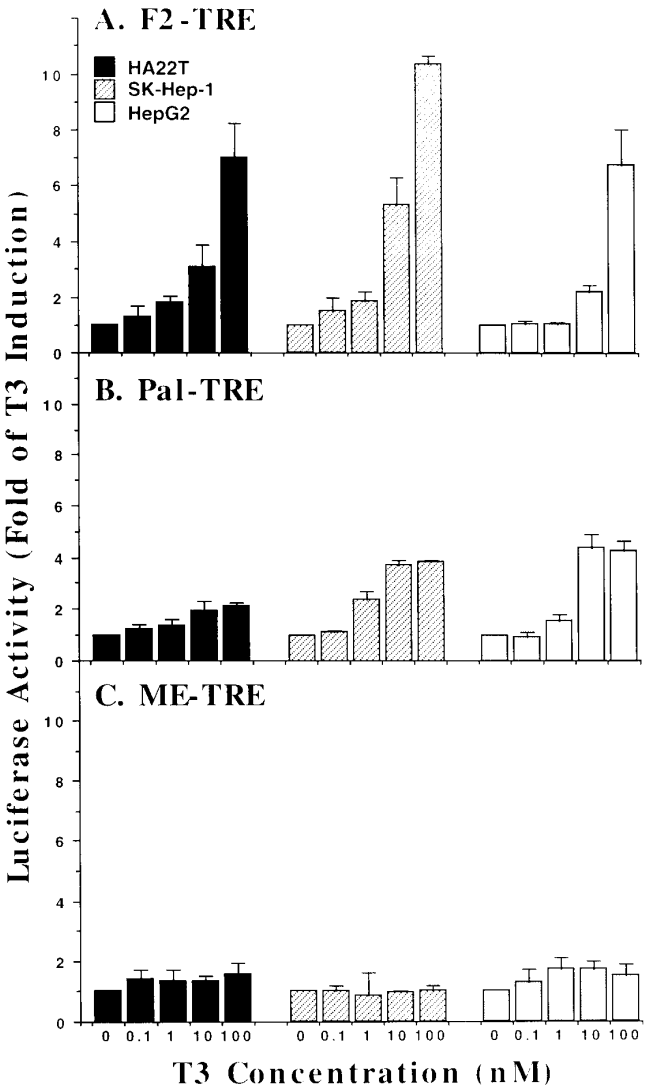

FIG. 4 T_3-dependent transactivation activity of TRs in HCC cells. HCC cells were transfected with luciferase reporter plasmid (2 μg) containing F2 in (A), Pal in (B), or ME in (C). The activity of luciferase was measured and normalized to the protein concentration of the lysates. The data were average of six independent experiments, each with duplicates (mean ± S. D., n=6).

tionally active, we first evaluated their transactivation activity by using reporter gene containing a F2-TRE, Pal-TRE or ME-TRE (-287 to -260 which consists of DR+4 TRE) as enhancers. Panels 4A-4C of Fig. 4 show that the endogenous TRs in the three HCC cell lines mediated the transactivation activity in a T_3-dependent manner, indicating that the TRs in these cells were transcriptionally active. Consistent with the previous findings reported for Cos-1 and NIH 3T3 cells (13), their transcriptional activities were TRE-dependent. In all these three cell types, the strength of TREs for promoting TR-mediated transcriptional activity was in the rank order of F2-TRE>Pal-TRE>ME-TRE.

Cell-type dependent regulation of the expression of endogenous ME gene by TRs in HA22T and SK-Hep-1. To understand whether the gene regulating activity of TRs on endogenous T_3 target genes was modulated by cell-type dependent factors, we evaluated the regulation of ME gene expression by TRs in HA22T and SK-Hep-1. ME gene is an important lipogenic gene in liver and its expression is known to be stimulated by T_3 and insulin (14). Furthermore, we have previously shown that the expression levels of TRβ1 and TRα1 proteins are identical in HA22T and SK-Hep-1 cells (15) so that it was possible to compare the TR-mediated effect on the expression of ME gene. Fig. 5 shows that T_3 stimulated the expression of ME (2.7 ± 0.2)- and (1.6 ± 0.1)-fold (mean ± SD; n) in HA22T and SK-Hep-1, respectively (bars 2 and 6, Fig. 5). However, insulin did not show differential stimulation, because a similar 1.5-fold stimulation was seen in these two cell lines (bars 4 and 8). Interestingly, the T_3-stimulated ME expression was repressed by insulin in HA22T cells (bars 2 vs. 3), but was additively stimulated by insulin in SK-Hep-1 cells (bars 6 vs. 7). These results suggest that cell-type specific factors not only modulated the extent of T_3-

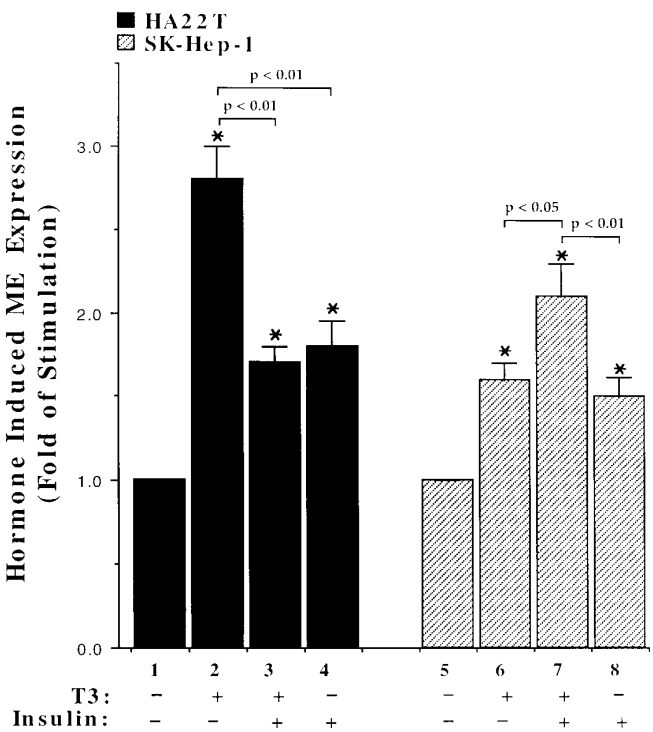

FIG. 5 Regulation of ME by T_3 and insulin. After cells were cultured in serum free medium for 24 hr., cells were incubated with fresh serum free media containing either 500 ng/ml insulin or 1 μM T_3. After 24 hr, cells were harvested, RNA was isolated, and Q-RT-PCR was performed using β-actin as an internal control as described in Methods. Data were expressed as means + S.D.(n). * represent that the differences (bar 2 vs. 3; bar 2 vs. 4; bar 6 vs. 7; and bar 7 vs. 8) were statistically significant. However, there was no statistical difference between bar 4 vs. 8.

stimulated ME expression, but also modulated the cross talk between TR- and insulin receptor mediated-pathways in HA22T and SK-Hep-1 cells. The regulation of the expression of ME gene could not be studied in HepG2 cells because no expression of ME was detected. At present, the reasons for no expression are not celar.

DISCUSSION

The present study characterized the TRs cloned from the three HCC cells, HA22T, SK-Hep-1 and HepG2. Their T_3 and TRE binding characteristics were indistinguishable from those of TRs isolated from other tissues (9, 10, 12). Using the reporter system, we found that these TRs could mediate T_3-dependent transactivation activity via the three TREs, indicating that these TRs were transcriptionally active.

The strength of TREs in promoting the transactivation of TRs was F2-TRE >Pal-TRE>ME-TRE in all three cell lines. On the same TRE, no significant differences in the transactivation activities of the TRs from these three cell lines were detected. However, when the gene regulating activities of these TRs on the endogenous ME gene were compared, a complex picture emerged. The TRs from HA22T showed approximately 2 two-fold stronger activity than TRs from SK-Hep-1 in stimulating the expression of ME gene, suggesting that the gene regulating activity of TRs was modulated by cell-type specific factor(s). These factor(s) probably required not only the ME TRE, but also the presence of its putative binding sites on the promoter region of ME gene. This notion was based on the findings that we did not detect different extents of transactivation activities between HA22T and SK-Hep-1 cells when only the ME TRE site was present in the reporter gene (see Fig. 4C). The cell-type specific factor(s) could also modulate the cross talk between the TR-and insulin receptor-mediated pathways. This notion was supported by the findings that the T_3-mediated stimulation on ME was inhibited by insulin in HA22T, but was enhanced by insulin in SK-Hep-1 cells. This opposite effect of insulin on the T_3-mediated ME stimulation was not due to the different activity mediated by insulin because the extent of insulin-stimulated ME gene expression was identical in HA22T and SK-Hep-1 cells (Fig. 5). The present results suggest that identical T_3-target genes present in these cells could be regulated by the cell type-specific factors leading to different functional consequences.

At the present time, the nature of the cell-type specific factor(s) which differentially modulated the TR-mediated regulation of ME gene in HA22T and SK-Hep-1 cells is unknown. However, the present study lends support to the emerging hypothesis that the diverse action of TRs requires the interplay of TRs with many cell-type specific factors. In the past, valuable information about pituitary-specific regulation of T_3-target genes has been obtained by using clonal strains of rat pituitary tumor growth hormone producing cell lines (16). However, despite the recognition of liver as a T_3-target tissue decades ago, no continuous liver cell lines are available for studying liver-specific regulation of T_3-target genes. The present study demonstrates the presence of functional TRs in these cell lines and the presence of TR interacting cell-type specific factors. Thus, these cell lines could serve as a model system to study the cell type-specific regulation of the T_3-target genes in liver.

REFERENCES

1. Cheng, S.-Y. (1995) *J. Biomed. Sci.* **2,** 77–89.
2. Mangelsdorf, D.J., and Evans R. M. (1995) *Cell* **83,** 841–850.
3. Lin, K.-H., Lin, Y.-W., McPhie, P., and Cheng, S.-Y. (1994) *Proc. XVI Int. Cancer Congress*, 615–622.
4. Cook, C. B., Kakucska, I., Lechan, R. M., and Koening, R. J. (1992) *Endocrinology* **130,** 1077–1079.
5. Sugimoto, T., Tsukamato, F., Fujita, M., and Takai, S. (1994) *Biochem. Biophy. Res. Comm.* **201,** 574–580.
6. Meier, C. A., Parkison, C., Chen, A., Ashizawa, K., Meier-Heusler, S. C., Muchmore, P., Cheng, S.-Y., and Weintraub, B. D. (1993) *J. Clin. Invest.* **92,** 1986–1993.
7. Lin, K.-H., Fukuda, T., and Cheng, S.-Y. (1990) *J. Biol. Chem.* **265,** 5161–5165.
8. Lin, K.-H., Zhu, X. G., Shieh, H. Y., Hsu, H. C., Chen, S. T., McPhie, P., and Cheng, S.-Y. (1996) *Endocrinology* **137,** 4073–4081. Comment in: Endocrinology (1996) 137, 4071–4072
9. Weinberger, C., Thompson, C. C., Ong, E., Lebo, R., Gruol, D. J., and Evans, R. M. (1986) *Nature* **324,** 18–25.
10. Nakai, A., Sakurai, A., Bell, G. I., DeGroot, L. J. (1988) *Endocrinology* **2,** 1087–1092.
11. Schueler, P. A., Schwartz, H. L., Strait, K. A., Mariash, C. N., and Oppenheimer, J. H. (1990) *Mol. Endocrinol.* **4,** 227–234.
12. Zhu, X. G., McPhie, P., Lin, K. H., Cheng, S.-Y. (1997) *J. Biol. Chem.* **272,** 9048–9054.
13. Wong, R., Zhu, X. G., Pineda, M. A., Cheng, S.-Y., and Weintraub, B. D. (1995) *Mol. Med.* **1,** 306–319.
14. Goodrige, A. G., and Adelman, T. G. (1976) *J. Biol. Chem.* **251,** 3027–3032.
15. Lin, K. H., Lin, Y.-W., Parkison, C., and Cheng, S.-Y. (1994) *Cancer Lett.* **85,** 189–194.
16. West, B. L., Catanzaro,, D. F., Mellon, S. H., Cattini, P. A., Baxter J. D., and Reudelhuber T. L. (1987) *Mol. Cell. Biol.* **7,** 1193–1197.