Triiodothyronine (T₃) activates rat liver S14 gene transcription through T₃ receptors (TRβ) binding distal thyroid hormone response elements located between −2.8 and −2.5 kilobase pairs upstream from the transcription start site. Previous studies suggested that proximal promoter elements located between −220 to −80 base pairs upstream from the 5′ end of the S14 gene were involved in hormone activation of the S14 gene. This report identifies an inverted CCAAT box (or Y box) at −104 ATTTG−100 as a core cis-regulatory element. Gel shift studies using rat liver nuclear proteins show that at least three CCAAT-binding factors interact with this region as follows: NF-Y and c/EBP-related proteins formed major complexes, whereas NF-1/CTF forms a minor complex in gel shift assay. Mutation of the Y box indicated that loss of NF-Y binding, but not c/EBP or NF-1, correlated closely with a decline in basal activity and a loss of T₃-mediated transactivation. Substitution of the S14 Y box in reporter genes with elements binding only NF-Y elevated basal activity and T₃-mediated transactivation, whereas substitution with elements binding c/EBP-related proteins or SP1 displayed low basal activity and T₃-mediated transactivation. These studies indicate that NF-Y and TRβ functionally interact to confer T₃ control to the S14 gene.

Thyroid hormone, *i.e.* triiodothyronine (T₃), receptors are members of the steroid/thyroid supergene family (1). T₃ receptors mediate changes in gene expression by binding thyroid hormone response elements (TREs) as heterodimers with the retinoid X receptor (RXR). Transfection and cell-free transcription studies have shown that artificial promoters containing a TATA box and hormone response element (HRE) are sufficient to achieve hormone-regulated transcription (2–4). T₃ receptors interact directly with the preinitiation complex (PIC) through general transcription factors (GTFs), like TFIIB or indirectly through co-activators, *e.g.*, CBP/p300 or co-repressors, *e.g.*, SMRT or N-CoR (5–9).

In natural promoters, however, nuclear receptor regulation of gene transcription is more complex. HREs are frequently found at a distance from the TATA box requiring chromatin folding to facilitate receptor-PIC interaction (10–15). Moreover, receptors functionally interact with other transcription factors, often binding in the vicinity of the HRE (12–24). Such interaction can synergistically induce or repress transcription. Regulation of the activity of these ancillary transcription factors can significantly impact overall promoter activity (16).

The rat liver S14 gene has served as a model for multifactorial regulation of hepatic gene transcription. Hepatic S14 gene transcription is induced by T₃, dietary carbohydrate, and insulin (14, 15, 25–36). Starvation, diabetes, polyunsaturated fatty acids, and elevation of intracellular cAMP suppress hepatic S14 gene transcription (26, 31, 32). T₃ induces mRNAᵢ₃ in liver, lactating mammary gland, and white adipose tissue but not cultured 3T3-L1 or 3T3-F442A adipocytes (25, 33, 34). Instead, glucocorticoids and retinoic acid replace T₃ as the major transcriptional activator of S14 in cultured fat cells (33, 34). In brain, heart, kidney, lung, spleen, testes, and pituitary, mRNAᵢ₃ is expressed at <1% of the liver level and is not regulated by T₃ (14). The cis-regulatory targets for the hormonal, nutritional, and tissue-specific regulation are located within two upstream enhancers and the proximal promoter. Three TREs are found within the thyroid hormone response region (TRR) located between −2.8 and −2.5 kb upstream from the transcription start site. Each TRE binds TR/RXR heterodimers and functionally interacts to confer T₃ control to the cis-linked gene (15, 25, 29, 30). The targets for carbohydrate, insulin, retinoic acid, and glucocorticoid control are found in a second enhancer located between −1.6 and −1.4 kb (28, 32). Since glucocorticoids and retinoic acid do not regulate hepatic S14 gene transcription, this region is designated a carbohydrate response region (CHO-RR).

In addition to these upstream enhancers, proximal promoter elements located between −220 and −80 bp upstream from the 5′ end of the gene have also been found to contribute to the tissue-specific expression of the gene (35, 36). This region is upstream from the transcription start site and a functional TATA box at −27/−21 bp and NF-1 site at −63/−48 bp. It binds tissue-specific factors that augment the rate of initiation of gene transcription (35, 36) as well as serving as a target for fatty acid-regulated transcriptional suppression (31). In this report, we have identified a key cis-acting element within this region and the transcription factor that binds this element. These studies show that this factor interacts functionally with T₃ receptors to control hepatic S14 gene transcription.

**EXPERIMENTAL PROCEDURES**

Construction of S14 Promoter Deletions—Construction of S14CAT fusion genes containing the full-length S14 promoter (−4315/+19) or

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1 The abbreviations used are: T₃, triiodothyronine; TR, thyroid hormone receptor; TRE, thyroid hormone response element; TRR, thyroid hormone response region; CHO-RR, carbohydrate response region; RXR, retinoid X receptor; GTF, general transcription factors; CFBI, CCAAT box binding factors; PIC, preinitiation complex; CAT, chloramphenicol acetyltransferase; HRE, hormone response element; bp, base pair(s); kb, kilobase pair(s); PCR, polymerase chain reaction; RLNP, rat liver nuclear extract; MLV, murine leukemia virus; RSV, Rous sarcoma virus.

2 A. Thelen and D. B. Jump, unpublished observations.
the S14TRR (−2.8/−2.5 kb) fused upstream from proximal promoter deletions (−290/+19; −220/+19; −120/+19; −80/+19; −40/+19) were described previously (31).

**Block and Linker-scanning Mutations within the S14 Proximal Promoter—**Block mutations within the S14 proximal promoter were constructed by using a modification of a two-step PCR-based approach (37). All oligonucleotides for PCR were synthesized by the MSU Biochemistry Department Macrostructure Facility. A block mutation was installed within the S14 proximal promoter by PCR to give a reporter plasmid containing the S14 promoter (−2900/+19) with a deletion from −69/−130 bp. The template plasmid contains +19 to −2900 bp within pCATAN, with an NsiI restriction site at −129 bp (Nat or S171). The NsiI site was added during the course of preparing linker-scanning mutants and does not inhibit S14CAT activity (see below and Fig. 2). The template was digested with NsiI and amplified (Perkin-Elmer, GeneAmp-XL-PCR Kit) using primers containing an NsiI site to generate the authentic sequence begins at −2900 bp. The 5′ primer DJ44 (antisense), 5′-ATATATTATGCATAT-3′, and DJ44 primer (see above) was used to synthesize a DNA fragment that was cloned into the NsiI/SphI site of the construction. The amplification of the two-step PCR-based method (37). Briefly, two PCR primers were synthesized to contain S14 sequences located at the 5′ and 3′ ends of the S14 gene along with the cloning sites, BamHI and XhoI, respectively. The S14CAT124 plasmid was used as template, and Vent polymerase was used for DNA synthesis via PCR. The 5′ primer (DJ29b(sense), 5′-TATTATTATGCATGATGGCTCAAAACAAGGCCGTGT; and DJ88, TTATATGTCCTTCCT(TTCAGAG) contains an artificial XhoI downstream from +19 bp. This 2930-bp fragment resulting from PCR was digested with BamHI and HindIII (cuts at −2111 bp) to generate a 819-bp fragment that was cloned into S14CAT126 containing sequences from −2111 to +19 bp. The result of this construction was a S14 promoter extending from −2930 to +19 bp cloned upstream from CAT. This construct contains the native S14 sequence.

**Linker-scanning mutations were created using two oligonucleotides containing an artificial NsiI (ATGCAT) site.** There are no NsiI sites within the S14 promoter (between −2930 and +19 bp). To construct a linker-scanning mutation between −119 to −130 bp, the 5′ sense primer (primers DJ63, AAAATGCATAT−102 bp) was used to synthesize a DNA fragment that was cloned into the NsiI/XhoI site of S14CAT126 (multicloning site: 5′-ATATATTATGCATAT-3′). A second fragment was synthesized using the 5′ sense primer DJ29 (see above) and the 3′ antisense primer (DJ64, AAAATGCATAT−102 bp) was used to synthesize a DNA fragment that was cloned into the NsiI/XhoI site of S14CAT126 (multicloning site: 5′-ATATATTATGCATAT-3′). The result of this second synthesis containing BamHI/XhoI ends was cloned into the plasmid containing the −119/+19 bp element. The result of this construction was the introduction of a 10− bp block mutation from −130 to −119 bp upstream from the 5′ end of the S14 gene, designated Mut1. Additional −10 bp mutations were made as follows: Mut 2, mutation from −111 to −102 bp using primers DJ87, ATATATTATGCATAT−102 bp; DJ88, TTATATGCATAT−102 bp; and DJ97, ATATATTATGCATAT−102 bp; and DJ98, TTATATGCATAT−102 bp. The resulting plasmid was then transfected with S14CAT126 containing sequences from −2111 to +19 bp. The result of this construction was a S14 promoter extending from −2930 to +19 bp cloned upstream from CAT. This construct contains the native S14 sequence.

**Construction of Linker-scanning Mutations—**Linker-scanning mutations within the S14 promoter were constructed using a modification of the two-step PCR-based method (37). Briefly, two PCR primers were synthesized to contain S14 sequences located at the 5′ and 3′ ends of the S14 gene along with the cloning sites, BamHI and XhoI, respectively. The S14CAT124 plasmid was used as template, and Vent polymerase was used for DNA synthesis via PCR. The 5′ primer (DJ29b(sense), 5′-TATTATTATGCATGATGGCTCAAAACAAGGCCGTGT; and DJ88, TTATATGTCCTTCCT(TTCAGAG) contains an artificial XhoI downstream from +19 bp. This 2930-bp fragment resulting from PCR was digested with BamHI and HindIII (cuts at −2111 bp) to generate a 819-bp fragment that was cloned into S14CAT126 containing sequences from −2111 to +19 bp. The result of this construction was a S14 promoter extending from −2930 to +19 bp cloned upstream from CAT. This construct contains the native S14 sequence.
Enhancer-Promoter Interaction

Deletion Analysis of the S14 Proximal Promoter—Fig. 1 illustrates the organization of functional elements known to control S14 gene transcription in rat liver. Previous studies have shown that elements between -220 and -80 bp of the transcription start site are important for overall promoter activity (31, 35). DNase I footprints of this region revealed a major site of DNA-protein interaction at -113/-88 bp (B-region) (35) and minor sites of interaction at -145 and -165 bp.² To localize the cis-acting elements that function in both the MLV-thyroid hormone receptor β1 expression vector (MLV-TRβ1). Cells were transfected using Lipofectin (Life Technologies, Inc.) at a ratio of 6.6 μg of Lipofectin to 1 μg of DNA. Two μg of CAT reporter gene and 1 μg of MLV-TRβ1 were added per plate. Some studies involved assessment of transfection efficiency using a luciferase expression vector (MamNedLuc, Clontee, Inc.). MamNedLuc was co-transfected with S14CAT/MLV-TRβ1 at 0.1 μg/well.

Cells were treated without and with T3 (1 μm) for 48 h with 1 ml media change after 24 h. Harvested cells were assayed for CAT and protein. Results are expressed as CAT activity in units (1 unit of CAT activity = 1 cpm 14C-acetylated chloramphenicol/h/100 μg protein) ± S.E, where the number of samples will be indicated in the figure. Expression of luciferase activity was measured using a Luciferase Assay System (Promega, Inc) and a Turner luminometer. Levels of luciferase activity ranged from 0.02 to 1.0 lumun units/100 μg of protein/min.

Gel Shift Analysis—Nuclear extracts used for gel shift analysis were prepared from rat liver (35, 36). Gel shift analysis using rat liver nuclear extracts was carried out using the following conditions: DNAs were ²P-labeled at the 5’ end using T3 polynucleotide kinase. Rat liver nuclear extract was mixed with ~5000 cpm of ²P-DNA in 25 mM Tris-Cl, pH 7.5, 40 mM KCl, 0.1% Nonidet P-40, and 10 mM glycerol, incubated for 20 min at room temperature, and electrophoresed in 8% acrylamide:bisacrylamide (75:1) gel at 200 V for 2 h, stained with ethidium bromide, and photographed under UV light.

RESULTS

Deletion Analysis of the S14 Proximal Promoter—Fig. 1 illustrates the organization of functional elements known to control S14 gene transcription in rat liver. Previous studies have shown that elements between -220 and -80 bp of the transcription start site are important for overall promoter activity (31, 35). DNase I footprints of this region revealed a major site of DNA-protein interaction at -113/-88 bp (B-region) (35) and minor sites of interaction at -145 and -165 bp.² To localize the cis-acting elements that function in both

FIG. 2. Effect of block and linker-scanning mutations within the proximal promoter on S14 gene transcription. The diagram illustrates the native (Nat, -2897/+19 bp) promoter and the location of a block mutation between -129/-69 bp (B1). The location of the five linker-scanning mutations are also illustrated as follows: MUT1 (-129/-120), MUT2 (-111/-102), MUT3 (-103/-94), MUT4 (-95/-86), and MUT5 (-79/-70). Primary rat hepatocytes were transiently transfected and treated with T3 as described in Fig. 1. Cells were co-transfected with MLVRPβ1 along with a S14CAT reporter gene: native (Nat1) S14CAT (-2897/+19), block (B1) or mutant (Mut 1-5) S14CAT fusion genes. After 48 h of T3 treatment cells were harvested for measurement of protein and CAT activity. The data are taken from two separate studies involving triplicate samples. The results are represented Fold Induction by T3 as mean ± S.E., n = 6.
basal and T₃-stimulated gene transcription, the S14 proximal promoter was sequentially deleted, and the S14TRR (−2.9/−2.5 kb) was placed immediately upstream from each truncated promoter construct. Primary rat hepatocytes were co-transfected with S14CAT fusion genes and a T₃-receptor expression vector (MLVTRβ1) to examine T₃ control of the S14CAT gene (31).

In the absence of T₃, basal S14CAT activity is very low in most constructs. Deletion of elements between −290 and −2500 bp leads to a ~70% decline in basal CAT expression and deletion to −80 or −40 bp leads to >95% decline in CAT activity. Analysis of luciferase activity in hepatocytes co-transfected with MamNeoLuc indicated that changes in CAT activity cannot be explained by differential transfection efficiency (not shown). The location of these deletions correlate with the presence of key cis-regulatory elements. Deletion of the −290/−2500-bp regions excises the CHO-RR and deletion of the −80 to −120-bp region excises the B-region of the S14 proximal promoter. The B-region was previously reported to represent a key cis-regulatory element involved in the hepatic-specific initiation of S14 gene transcription (35, 36).

In the presence of T₃, the full-length S124 plasmid (−4315/−19 bp) is induced 50-fold (from 127 ± 27 to 6396 ± 740 units). Moving the TRR closer to the proximal promoter increases the level of T₃-mediated transactivation to 140-fold (S156). However, the constructs containing the NF-1 and the PIC sites (S158 or S159) show the same response to T₃ as the full-length plasmid. Because the basal activity is reduced by >95% in the S158 and S159 constructs when compared with the full-length construct, the overall CAT activity is similarly reduced, i.e. from ~6400 CAT units in S124 to ~250 units in S158 and S159. These findings indicate that proximal promoter elements affecting basal activity significantly impact the overall level of T₃-stimulated S14CAT activity. The cis-acting elements within the proximal promoter region mediating the greatest change in both basal and T₃-stimulated expression are located between −120 and −80 bp.

**Block and Linker-scanning Mutations of the −120/−80-bp Region**—The promoter deletion studies described in Fig. 1 placed the S14TRR immediately adjacent to the proximal promoter region. Because the S14 TRR is normally at −2.5 to −2.8 kb such a change in position may not accurately reflect the role proximal promoter elements play in hormonal control of the S14 gene. Consequently, a block mutation of the B-region was installed to assess its effect on transcription of this gene (Fig. 2). The full-length native S14CAT construct extends from −2897 bp to −19 bp (Nat1) and is induced 54-fold by T₃. However, deletion of the −130/−69-bp region (B11) results in low basal CAT activity (31 ± 13 units) and a marginal (45%) augmentation following T₃ treatment.

Because the B11 mutation spanned nearly 60 bp and the footprinted region (35) spanned ~25 bp, a linker scanning approach was used to localize better the key cis-regulatory elements. Accordingly, five linker-scanning mutations of −10 bp in length were made extending from −129 to −70 bp (Fig. 2). Although mutations flanking the footprinted region (−113/−88 bp), i.e. Mut1 (−129/−120 bp) and Mut5 (−79/−70 bp) induced marginal changes in S14 CAT activity, mutations within the footprinted region had a significant effect on S14CAT activity. Mut2 (−111/−102 bp) and Mut3 (−103/−94 bp) showed very low CAT activity in the presence of T₃ (10 ± 3 and 70 ± 14 CAT units, respectively), whereas Mut 4 (−95/−86) displayed an 8-fold response to T₃. These studies suggest that the region between −111 and −94 bp is critical for both basal and T₃-mediated activation of S14CAT activity. Co-transfection with MamNeoLuc indicated that these differences were not attrib-
tuated to changes in transfection efficiency (not shown).

Mutation Analysis of the −120/−80 bp—During the course of our studies, we discovered that placing the S14TRR immediately upstream from the RSV basal promoter (−60/+20 bp; R127), which contains only a TATA box (39), led to a marginal 3-fold increase in CAT activity following T3 treatment (Fig. 3). Inserting the S14 proximal promoter elements (−220/−80 bp) led to a marginal 3-fold increase in CAT activity compared with R127. Replacing the S14 TRR in R117 with a 20-bp insert significantly increased basal activity but had no effect on T3-mediated transactivation when compared with R127. Replacing the S14 TRR in R117 with a canonical TRE (a tetramer of a DR + 4; R126) confers the same level of T3-mediated transactivation (12-fold) as seen with R117. However, if the −120/−80-bp region is deleted from this construct, the level of transactivation is comparable to that seen with R127, a construct containing no −120/−80 element (Fig. 3). The M4 and M5 mutations span the sequence −120ATTGGGC−99, which is an inverted CCAAT box or Y box.

DNA Sequence and Gel Shift Analysis of the B-region—Fig. 5A illustrates the DNA sequence for the B-region (−113/−88 bp), the inverted CCAAT box at −104/−100 bp, the location of the linker scanning, and 3-bp mutations. Gel shift analysis was used to identify proteins binding this region. Rat liver nuclear proteins (RLNP) bind 32P-B-region and form five slow migrating bands with increasing protein concentrations (Fig. 5B). Competition with a 100-fold molar excess of the native (N) sequence eliminates all shifting, whereas competition with oligonucleotides corresponding to the Mut2 (M2) and Mut3 (M3) mutations (see Fig. 2) show partial competition for bands 1–3 and no competition for bands 4 and 5. No competition was detected using a thyroid hormone response element (DR + 4). This pattern of competition along with the functional studies (Fig. 2) suggest that factors leading to the formation of bands 4 and 5 might be important for S14 promoter function.

Better resolution of the elements required for the formation of the specific complexes was obtained by using the oligonucleotides containing 3-bp mutations (Fig. 4). Competition gel shift analysis (Fig. 5C) with native (N) and mutant (m1, m2, m3, m6, and m7) oligonucleotides showed nearly identical competition patterns, i.e. all five bands were lost at a 100-fold competitor concentration. In contrast, oligonucleotides m4 and m5 failed to compete for the formation of bands 4 and 5. Oligonucleotide m4 competed fully for band 1 and partially for
NF-1 binding (band 1) was competed by all mutations. The lack of correlation between NF-1 binding and abrogated T₃-mediated transactivation (Figs. 4 and 5) suggested that NF-1 binding does not play a role in S14 promoter function at the Y box. Accordingly, our studies focused on NF-Y and c/EBP binding to the B-region. Specific antibodies were used in gel shift assays (Fig. 7). NF-Y is a heterotrimeric transcription factor composed of three subunits, A, B, and C (40). Treatment of RLNP with antibodies directed against either the A- or B-subunit led to a decline in the formation of bands 4 and 5 and the formation of slower migrating “supershifted” bands. In contrast, addition of an anti-ARP-1 antibody did not consistently affect bands 4 or 5. Based on this analysis, both bands 4 and 5 contain the A- and B-subunits of NF-Y.

A similar approach was used to examine the association of c/EBP with the S14 B-region (Fig. 7B). In the samples labeled Nativ, either anti-c/EBPα, anti-c/EBPβ, or anti-ARP-1 antibodies were added to rat liver nuclear proteins. Although anti-c/EBPα-depleted band 2 only, no supershifted product was detected. Anti-c/EBPβ or anti-ARP-1 antibody did not affect the mobility of any band. This observation suggests that band 2 may contain c/EBPa. To better resolve this issue, we took advantage of the fact that c/EBPs are heat-stable DNA-binding proteins (44). Heating rat liver nuclear proteins to 70 °C for 10 min prior to adding DNA leads to a loss of bands 1, 4, and 5, indicating that these factors are heat-sensitive. NF-1 and NF-Y are heat-labile proteins (40, 42).

For comparison, the m5 mutation (Figs. 4 and 5) failed to compete for bands 4 and 5. This profile of competition suggests that formation of band 1 is due to NF-1; band 2 and possibly 3 is due to c/EBP (or related proteins), and bands 4 and 5 form when NF-Y binds.

NF-1 binding (band 1) was competed by all mutations. The lack of correlation between NF-1 binding and abrogated T₃-mediated transactivation (Figs. 4 and 5) suggested that NF-1 binding does not play a role in S14 promoter function at the Y box. Accordingly, our studies focused on NF-Y and c/EBP binding to the B-region. Specific antibodies were used in gel shift assays (Fig. 7). NF-Y is a heterotrimeric transcription factor composed of three subunits, A, B, and C (40). Treatment of RLNP with antibodies directed against either the A- or B-subunit led to a decline in the formation of bands 4 and 5 and the formation of slower migrating “supershifted” bands. In contrast, addition of an anti-ARP-1 antibody did not consistently affect bands 4 or 5. Based on this analysis, both bands 4 and 5 contain the A- and B-subunits of NF-Y.

A similar approach was used to examine the association of c/EBP with the S14 B-region (Fig. 7B). In the samples labeled Nativ, either anti-c/EBPα, anti-c/EBPβ, or anti-ARP-1 antibodies were added to rat liver nuclear proteins. Although anti-c/EBPα-depleted band 2 only, no supershifted product was detected. Anti-c/EBPβ or anti-ARP-1 antibody did not affect the mobility of any band. This observation suggests that band 2 may contain c/EBPa. To better resolve this issue, we took advantage of the fact that c/EBPs are heat-stable DNA-binding proteins (44). Heating rat liver nuclear proteins to 70 °C for 10 min prior to adding DNA leads to a loss of bands 1, 4, and 5, indicating that these factors are heat-sensitive. NF-1 and NF-Y are heat-labile proteins (40, 42).

Addition of the c/EBPa antibodies to the nuclear proteins after heating/cooling led to a selective depletion of bands 2 and 3 and a shift to a slow migrating position coincident with band 4 (Native). c/EBPβ antibodies also produced a faint supershifted band; however, the origin of this shift cannot be determined. ARP-1 antibodies did not induce consistent changes in band intensity or mobility. These results support the competition binding studies by showing that c/EBPa and c/EBPβ bind the S14 B-region to form band 2 and possibly band 3.

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NF-1, but Not c/EBP-related Proteins, Augments S14 Promoter Activity—The gel shift studies indicate that both NF-Y and c/EBP-related proteins bind the S14 B-region. To determine which factor is functional within the S14 promoter, the B-region in the R117 reporter gene was replaced with either the albumin C-region, the albumin D-region, or a composite element derived from the thymidine kinase promoter containing binding sites for Sp1 and NF-Y (Fig. 8). Substitution of the albumin C element both augments basal CAT activity and stimulates T₃-mediated transactivation comparable to the RSVCAT reporter gene containing the native S14 B-region (R117). In contrast, substitution of the albumin D element led to a decline in both basal activity and T₃-mediated transactivation. The composite element containing two Sp1 sites flanking an NF-Y site (Sp1-NFY-Sp1: SNS) was nearly identical to the construct containing the native S14 B-region. Substitution of a single Sp1 element failed to induce promoter activity (not shown). Based on this analysis, c/EBP-related factors and Sp1 do not function in the context of the S14 proximal promoter. In contrast, elements binding NF-Y either alone (i.e. Alb-C) or in combination with other elements (i.e. SNS) augment both basal and T₃-mediated transactivation of the S14 gene. These studies indicate a specific requirement for NF-Y within the S14 proximal promoter.
DISCUSSION

We previously reported that elements within the S14 proximal promoter B-region (−157/−88 bp) were important for tissue-specific regulation of hepatic S14 gene transcription (35, 36). In this report, a combination of deletion and block mutations along with linker analysis and 3-bp mutations was used to localize a key cis-regulatory element within this region to an inverted CCAAT box, 2105GATTGGC (or Y box). Mutations within this element lower basal activity and essentially abrogate T3-mediated transactivation of the S14 gene (Figs. 2–4). The Y box and immediate flanking sequences (−2107GG-GATTGG CTCAAAACAAG−89) show 80% identity to an NF-Y consensus sequence (CTGATTGGYY), 44% identity to a c/EBP consensus sequence (ATTGCNNAA), and 83% identity to a NF-1 palindromic recognition site (TTGGCTN 3AGCCAA, reverse) (20, 45–48). At least three CBPs, i.e. NF-Y, NF-1, and c/EBP-related proteins, bind this element (Fig. 5–7). However, only NF-Y augments basal activity and stimulates T3-mediated transactivation. The Y box, together with the distal TREs, forms a functional T3-regulatory unit. The functional synergy between the TREs and the Y box indicates that proximal promoter elements play an important role in hormone-activated gene transcription.

NF-Y (also known as CBF, CP1, and YEBP) is a heterotrimeric transcription factor composed of three subunits (A, 42

FIG. 6. NF-Y, c/EBP, and NF-1 bind the S14 B-region. A, the DNA sequence used in competitive gel shift analysis. The S14 B-region sequence along with the albumin C, albumin D elements, c/EBP, NF-1, and SP1 consensus sequences are shown. B, RLNP (4 μg) was used to shift 32P-labeled B-region as described in Fig. 5. Non-radioactive competitor oligonucleotides were added at a 100-fold molar excess. The competitors were albumin C-region (Alb-C), albumin D-region (Alb-D); a consensus c/EBP, NF-1, and SP-1 (Santa Cruz Biotechnology) and M5, Fig. 4. Other studies established that thyroid hormone receptors, AP1, AP2, AP3, NF1b, SP1, RARα, PPARα, and HNF-4 did not bind the B-region. The results are representative of at least three gel shift assays.

FIG. 7. Antibodies to NF-Y and c/EBP interact with proteins binding the B-region. A, NF-Y antibodies (Rockland Immunobiochemicals) were used to test NF-Y association with the B-region. RLNP (2 μg) was incubated with 5 μg of antibodies to the NF-Y A-subunit (NF-YA), B-subunit (NF-YB), or ARP-1 for 2 h on ice. The m5 oligonucleotide was added to enhance formation of bands 4 and 5. 32P-Labeled B-region was added, and after 20 min at room temperature, the preparation was electrophoretically separated. The apparent decline in bands 4 and 5 intensity following ARP-1 antibody treatment is not seen consistently, e.g. see ARP-1 effects in B or Fig. 7. NF-Y antibodies had no consistent effect on the formation or mobility of bands 1–3 indicating specificity of the NF-Y antibodies used in these studies. B, c/EBP antibodies (Santa Cruz Biotechnology) were used to examine the association of c/EBPα and β with the S14 B-region. Native (Native) and heated (70 °C, 10 min) RLNP (4 μg) were reacted with 2 μg of antibody against c/EBP α (c/EBP-A), c/EBP β (c/EBP-B), or ARP1, and the products were separated as described above. The autoradiogram for the Native and Heated 70 °C gel shifts were exposed for 1 and 2 days, respectively. These supershift studies are representative of three separate studies.
NF-Y, but not c/EBP, can substitute for the S14 B-region in the S14 promoter. The S14 B-region in R117 (S14-B) was replaced with either the albumin C (Alb-C), albumin D (Alb-D), or and composite element from the thymidine kinase promoter containing 2-SP1 flanking an NF-Y site (SNS). After transfection, cells were treated with T3 as described in Fig. 3. Open bars, no T3 treatment; closed bars, T3 treatment. Results are representative of two separate experiments with triplicate samples. Mean ± S.E., n = 6.

NF-Y shows a wide tissue distribution and is highly conserved. The A- and B-subunits show homology to the yeast CCAAT box binding transcription factors HAP 3 and 2, respectively. Formation of a complex between the A- and C-subunits is required to bind the B-subunit, and together, the heterotrimeric complex binds DNA (57). The B-subunit has a glutamine-rich area and a serine/threonine-rich region on the amino-terminal end; both are required for full transcriptional activation (54). The hydrophilic carboxyl-terminal end is rich in basic residues and contains distinct subunit interaction and DNA binding domains.

NF-Y binds CCAAT boxes located within ~100 bp of the transcription start sites, and these elements are known to be important for early functions in preinitiation complex formation (43, 49). The B-subunit may interact with the preinitiation complex through TAF110 (54). NF-Y has also been reported to interact with transcription factors binding upstream elements. For example, c/EBPα and NF-Y both bind within a 40-bp element of the albumin promoter. These factors functionally interact to enhance the rate of initiation of albumin gene transcription (43). Similar results have been reported for the major histocompatibility complex DRA and Ii promoters (46, 52, 56). Although these studies implicate a functional interaction between NF-Y and other transcription factors, a specific role for NF-Y in transcription remains to be established.

The interaction between NF-Y and other transcription factors is reminiscent of the role ancillary factors play in nuclear receptor activation. Nuclear receptors interact directly with the TRE and Y box. Since other T3-responsive genes do not require a Y box, this suggests that a functional S14 T3 response unit consists of the TRE and Y box. Since other T3-responsive genes do not contain Y boxes, for example, the phosphoenolpyruvate carboxykinase promoter, the NF-Y/Y box is likely not a general requirement for S14 gene transcription. Some clue to the role of the Y box in S14 gene transcription may be the fact that TRR and the Y box are separated by >2.5 kb. Such a separation argues against cooperative binding and implicates a requirement for chromatin looping to bring the S14 TRR into juxtaposition to the TATA box so that TRβ/RXR heterodimers can interact with GTFs. The Y box is situated within a DNase I-hypersensitive site that forms just prior to hepatic S14 gene activation during post-natal development (58). T3 administration to the pre-weaned rat cannot activate this gene prior to weaning despite the presence of functional T3 receptors (59). Whether NF-Y functions to maintain this open chromatin structure or facilitates TR/RXR-GTFs interaction will require additional experimentation.

An important outcome of these studies was the finding that T3-mediated transactivation could be controlled by the composition of the proximal promoter region. The S14 proximal promoter is a target for tissue-specific factors that augment hepatic gene transcription (35, 36) and fatty acid-regulated factors that attenuate S14 gene transcription (31). The Y box and NF-Y are key functional elements within this region. The ubiquitous tissue distribution of NF-Y cannot account for the specific augmentation in the initiation of S14 gene transcription.
transcription (35, 36). Moreover, NF-Y, per se, is likely not the target of fatty acid control because fatty acids do not suppress TK promoter activity, a promoter binding NF-Y (31). Preliminary studies have indicated that elements upstream from the Y box are also important for S14 gene transcription. We speculate that these upstream elements bind tissue-specific factors that together with NF-Y will be important for tissue-specific and fatty acid-regulated control of S14 gene transcription.

In conclusion, T₃-mediated transactivation of the S14 gene requires NF-Y as a crucial factor binding the S14 proximal promoter. Despite the fact that the TR/RXR and NF-Y binding sites are separated by >2.5 kb, NF-Y and TR/RXR functionally interact to confer T₃ control to the hepatic S14 gene. Although several CBFs bind this element, only NF-Y was found to enhance both basal and T₃-stimulated transactivation. This represents the first report of NF-Y participation in T₃-regulated gene transcription. Further experimentation should clarify the role NF-Y plays in these processes.

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REFERENCES

The CCAAT Box Binding Factor, NF-Y, Is Required for Thyroid Hormone Regulation of Rat Liver S14 Gene Transcription
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