NF-Y Has a Novel Role in Sterol-dependent Transcription of Two Cholesterogenic Genes*

(Received for publication, July 17, 1995)

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The transcription of farnesyl diphosphate (FPP) synthase is regulated up to 30-fold by the sterol status of the cell. Point mutations in a 6-base pair ATTGGC sequence in the promoter disrupt both sterol-dependent transcription in vivo as well as binding of the transcription factor NF-Y in vitro. Co-transfection of cells with NF-YA29, a dominant negative form of NF-Y, and various promoter-reporter genes specifically inhibits the sterol-dependent regulation of FPP synthase and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase. In contrast, NF-YA29 does not affect the regulation of reporter genes under the control of promoters derived from either the HMG-CoA reductase or the low density lipoprotein receptor gene. Transient expression of the 68-kDa transcriptionally active fragment of sterol regulatory element-binding protein in cells stimulates an HMG-CoA synthase-reporter gene over 90-fold. This induction is blocked in cells co-expressing NF-YA29. We hypothesize that NF-Y plays a novel role in sterol-dependent regulation of two key genes in the cholesterol biosynthetic pathway and that this role requires a specific interaction with the sterol regulatory element-binding protein or related transcription factors.

FPP synthase catalyzes the synthesis of FPP, a key isoprenoid required for the synthesis of sterols, dolichol, ubiquinone, heme a and geranylgeranyl diphosphate, for the prenylation of a number of proteins including Ras, and for the synthesis of farnesol, a new regulatory molecule (1–3). Therefore, this gene encoding FPP synthase, like those encoding the LDL receptor, HMG-CoA synthase and HMG-CoA reductase, is regulated by the cholesterol status of the cell; transcription is high in cells deprived of sterols and low in cells exposed to excess sterols (4–7). The detailed mechanisms involved in the coordinate regulation of all these genes by sterols have not yet been established. However, recent studies have demonstrated that the promoters of HMG-CoA synthase and the LDL receptor contain a 10-bp sterol regulatory element (SRE-1) which functions as a binding site for a family of positive transcription factors termed SRE-binding proteins (SREBP) (7–10). Point mutations within the SRE-1 of the LDL receptor disrupt SREBP binding in vitro and sterol mediated transcriptional regulation in vivo (10). Two hamster SREBP genes have been identified, which encode five proteins as a result of differential splicing (10). However, both SREBP-1 and SREBP-2 bind the LDL receptor SRE-1 and activate transcription (7–10). Osborne et al. recently demonstrated that a distinct protein, Red 25, is involved in sterol-dependent regulation of the HMG-CoA reductase gene through interaction with a sequence that partially overlaps an SRE-1 (11). In contrast, a reporter gene driven by 117 bp of the FPP synthase promoter is regulated 25-fold in response to sterols, even though there are no nucleotide sequences with high homology to either the SRE-1 or the Red 25 binding sites (5).

Recently we reported that mutagenesis of the 6-base pair sequence ATTGGC (an inverted CCAAT box) in the rat FPP synthase promoter impaired sterol-dependent transcription of this gene (5). In the current report we have used gel shifts and gel supershift assays to identify the protein that binds to the inverted CCAAT box present in the promoters of both FPP synthase and HMG-CoA synthase. The physiological importance of this transcription factor, namely NF-Y, in the sterol-dependent transcription of both FPP synthase and HMG-CoA synthase was confirmed in vivo following transfection of cells with a plasmid encoding a dominant negative form of NF-YA.

EXPERIMENTAL PROCEDURES

Materials—DNA restriction and modification enzymes were obtained from Life Technologies, Inc. Oligonucleotides were obtained from Dr. D. Glitz (Department of Biological Chemistry, UCLA) and end-labeled by standard procedures (12). Lipoprotein-deficient fetal calf serum was obtained from PerImmune. The sources of all other reagents have been given previously (4, 5).

Plasmid Construction and Mutagenesis—Standard molecular biology techniques were used (12). Fragments (247 bp) of the FPP synthase promoter (nucleotides −47 to −293) (4, 5) containing either wild-type ATTGGC (wt) or transversions CGGTTA (mut2) were subcloned into the pGL2 basic Vector (Promega). Single point mutations in the ATTGGC sequence were prepared in pFPPS-0.247 utilizing the Sculptor mutagenesis kit (Amersham Corp.) (5) prior to subcloning into pGL2, the luciferase plasmid. All mutations were confirmed in pGL2.

The luciferase reporter gene pFPPS-0.319L contained 319 bp of the FPP synthase promoter excised from pFPPS-0.319 (5) and inserted into pGL2. pSYN SRE contained nucleotides −324 to −225 of the promoter of HMG-CoA synthase fused to the HMG-CoA synthase TATA box and inserted into pGL2 (13). The construction of pDL SRE, which contains three repeats of an SRE-1 sequence and an adjacent Sp1 binding site, has been reported (14). pRed CAT-1 contains nucleotides −277 to +231 of the HMG-CoA reductase gene upstream of the CAT reporter gene (15). KS5CAT, which contains the CAT gene under the control of the SV40 promoter and pCMV β-galactosidase, which encodes β-galactosidase under the control of the CMV promoter, have been reported previously (4, 5). The expression plasmid pCMV-CSA10 was obtained by insertion of a BamHI-HindIII fragment into a BglII-HindIII-cut
pCMV5. The inserted fragment encodes the first 490 amino acids of SREBP-1a and was generated by PCR using HepG2 mRNA and oligonucleotides containing the above restriction sites. The expression of the 68-kDa aminoterminal domain of SREBP was under the control of a CMV promoter, pNF-Y29, a generous gift from Dr. Roberto Mantovani, contains a mutated NF-YA (16) under the control of the SV40 promoter.

Cells and Transfection—CHO cells were transfected with the luciferase plasmids together with β-galactosidase and pSV2neo and stable transformants were obtained as described previously (4, 5). Duplicate dishes of G-418-resistant cells were incubated for 20 h in media containing 10% lipoprotein-deficient fetal calf serum (LPDS), 5 μM mevinolin and 0.5 mM mevalonic acid in the absence (inducing) or presence (repressing) of cholesterol and 25-hydroxycholesterol (10 and 1 μg/ml), respectively (5). Luciferase activities in lysed cells were normalized for β-galactosidase activity (4, 5).

CV-1 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C. Duplicate dishes of cells were each transiently transfected with a total of 7 μg of plasmid DNA/60-mm dish using the Stratagene MBS transfection kit.

Transfections contained 4 μg of the reporter gene, 2 μg of β-galactosidase plasmid and pNF-YA29 and/or plasmid encoding SREBP (pCMV-UA30) as indicated in the text. Carrier DNA (KSoCAT) was used as required. Following transfection, the cells were incubated for 20 h under inducing or repressing conditions, as indicated in the legend.

Gel Mobility Shift Assays—Double-stranded DNA corresponding to either nucleotides –298 to –272 of the FPP synthase promoter (4) or nucleotides –324 to –225 from the HMG-CoA synthase promoter were end-labeled, gel-purified, and used in gel shift studies as described previously (4, 5). The probe (20,000 cpm, 1.5 fmol) was incubated at 20 °C for 60 min with the indicated amount of CHO nuclear extract (1 or 5 μg). Where indicated, 0.2 μg of antibody to a specific CCAAT box-binding protein was then added, and the incubation continued for 30 min at 4 °C prior to electrophoresis.

RESULTS AND DISCUSSION

Mutagenesis of the 6-base pair sequence ATTGGC in the FPP synthase promoter disrupted sterol-dependent transcription of reporter CAT or luciferase genes (4, 5). The transcription factor that bound to this site was not identified. Fig. 1A demonstrates that single point mutations at A, T, T, G, or G within FPP synthase promoter-reporter genes stably transfected into CHO cells resulted in the loss of sterol-dependent transcription equivalent to that seen when all 6 bp were mutated; the fold regulation declined from 4-fold to less than 1.5-fold. Mutation of the sixth nucleotide (C → A) resulted in intermediate regulation (Fig. 1A). While mutagenesis of the inverted CCAAT box of many other genes results in a drastic decline in basal transcriptional rates (17), no such effect was observed for FPP synthase (5) (data not shown) (2).

A number of distinct transcription factors have been identified that bind to an inverted CCAAT box or to related sequences (18). In order to identify the specific protein that binds to the ATTGGC sequence in the FPP synthase promoter, a 27-bp radiolabeled oligonucleotide was used in electromobility shift assays. Incubation of this probe with nuclear proteins produced two shifted complexes, which were competed by either excess unlabeled wild-type probe or a probe containing a mutation in the sixth nucleotide (C → A) (Fig. 1B). Probes containing point mutations at A, T, T, G, or G were ineffective or poor competitors (Fig. 1B). Fig. 1C demonstrates that the complex obtained with the wild-type probe was supershifted by antibodies that recognize specific subunits of NF-Y but not by antibodies that recognize CBF-HSP (19), a distinct CCAAT box-binding protein; anti-NF-YA and anti-CBF-A recognize two distinct monomers, termed NF-YA and NF-YB, respectively, in humans, which form a CCAAT box-binding multimer (16, 20).

A similar supershift result was obtained using a 100-bp radiolabeled probe of the HMG-CoA synthase promoter containing

two SRE-1 sequences and an inverted CCAAT box (Fig. 1D). Preliminary results indicate that mutation of the A in the ATTTGGC sequence of an HMG-CoA synthase promoter-reporter plasmid (pSVE SRE) disrupts sterol-dependent regulation.

Taken together, the results in Fig. 1 indicate that NF-Y binds to the inverted CCAAT box in the FPP synthase and HMG-CoA synthase promoters in vitro. Moreover, the same nucleotides that are important for this interaction are also required for sterol-dependent transcriptional regulation in vivo.

NF-Y is a ubiquitously expressed multi-component factor that is required for activation of several genes that are expressed in a tissue-specific fashion (21). The observation that, in these cases, NF-Y is not itself the tissue-specific factor indicates that it functions together with an appropriate tissue-specific regulatory protein to establish the correct pattern of expression. In the case of the albumin promoter, NF-Y interacts with the liver enriched factor hepatic nuclear factor-1 (22) and in the case of the major histocompatibility complex II genes, NF-Y functions synergistically with the X-box DNA-binding protein and CIITA, a novel non-DNA-binding protein (23).

Mantovani et al. (16) recently demonstrated that NF-YA29, a dominant negative form of NF-Y in which three amino acids in the DNA binding domain have been mutated, forms a complex with NF-YB. This complex was functionally inactive in an in vitro transcription assay (16). In order to determine whether NF-Y has a physiological role in sterol-dependent transcription of the FPP synthase and other sterol-regulated genes, we utilized a plasmid encoding this mutant (pNF-YA29) in cotransfection experiments with various reporter genes. Co-transfection of CV-1 cells with pNF-YA29 and either FPP synthase (pPPS-0.319L) or HMG-CoA synthase (pSVE SRE) reporter genes decreased the maximal induced reporter enzyme levels by over 80% (Fig. 2, A and B, open bars). NF-YA29 expression also significantly decreased the fold regulation of these reporter genes in response to sterols (Fig. 2, A and B). These effects were specific since co-expression of pNF-YA29 with plasmids containing reporter genes under the control of promoters derived from the LDL receptor (pLDL SRE), HMG-CoA reductase (pRed CAT-1), SV40 (pSVE CAT), or CMV (pCMV-β-G) had only limited effects on expression or regulation of these reporter genes (Fig. 2, C-F).

The data presented in Fig. 2 predict that expression of NF-YA29 in CV-1 cells should result in a decreased concentration of transcriptionally active NF-Y. This prediction is supported by the data shown in Fig. 3. The amount of shifted complex obtained with a radiolabeled FPP synthase probe containing ATTTGGC was decreased significantly when the nuclear extract was obtained from cells transiently transfected with NF-YA29 as compared to non-transfected cells (Fig. 3A, lane 2 versus lane 3). This decrease was specific since the complex obtained with a probe recognized by the ubiquitous transcription factor Oct-1 was unaffected by prior transfection of the cells with pNF-YA29 (Fig. 3B, lane 5 versus lane 6). These results demonstrate that the amount of transcriptionally active nuclear NF-Y available to bind to the ATTTGGC sequence is decreased following NF-YA29 transfection even under transient transfection conditions where all cells do not contain the mutant NF-YA29 protein. The observed decrease in functional nuclear NF-Y levels is consistent with both the impaired regulation of reporter genes under the control of FPP synthase and HMG-CoA synthase promoters (Fig. 2) and with the finding of Mantovani et al. that the mutant NF-YA29 subunit can sequester NF-YB into defective complexes (16).

Taken together, the results in Figs. 2 and 3 are consistent with a functional role for NF-Y in the sterol-dependent transcriptional regulation of both the FPP synthase and HMG-CoA synthase genes.

The sterol-dependent regulation of HMG-CoA synthase is known to require SREBP (7). The results presented above indicate that NF-Y also has a role in this regulation. We hypothesize that sterol-mediated regulation may depend on a functional interaction of NF-Y with either SREBP or a related transcription factor since this would provide sterol-mediated specificity. If this hypothesis is valid, introduction of NF-YA29,
the dominant negative form of NF-YA, into cells would be expected to interfere with sterol-dependent regulation of genes requiring both NF-Y and SREBP even in the presence of excess SREBP.

In order to test this hypothesis, we have utilized an expression plasmid that encodes a 68-kDa transcriptionally active fragment of SREBP under the control of a CMV promoter. Overexpression of this SREBP fragment induces the transcription of HMG-CoA synthase in a sterol-independent manner (Fig. 4, lanes 5 and 6 versus 1 and 2) consistent with the studies of Hua et al. (7). As expected, NF-YA29 co-expression blocked the induction of the HMG-CoA synthase reporter gene, which is normally observed in cells incubated in media deprived of sterols (Fig. 4, lanes 3 and 4 versus 1 and 2). In addition, the induction mediated by the 68-kDa fragment of SREBP, which was observed in the absence or presence of sterols, was also blocked by NF-YA29 co-expression (Fig. 4, lanes 7 and 8 versus 5 and 6). We conclude that expressed NF-YA29 has a direct inhibitory effect on the transcription of the HMG-CoA synthase gene as a result of the formation of functionally inactive NF-Y within the nucleus. We propose that a similar mechanism accounts for the effect of the dominant negative NF-YA29 on the transcription of FPP synthase promoter-reporter genes in vivo (Fig. 2).

The results are consistent with a novel physiological role for NF-Y in sterol-dependent transcriptional regulation of the FPP synthase and HMG-CoA synthase genes. The observation that the ubiquitous transcription factor NF-Y is important for sterol-dependent regulation of FPP synthase suggests that a separate DNA sequence and transcription factor provides additional specificity.

The current studies demonstrate that NF-Y participates in a nutritional regulatory response for at least two sterol-regulated genes. It is not the regulatory protein itself, but it is essential for optimal activation. As such, its role may be similar to that of the ubiquitous transcription factor Sp1 in sterol-dependent regulation of the LDL receptor gene (14, 24). In these latter studies, DNA-bound SREBP promoted the binding of Sp1 to an adjacent site and resulted in transcriptional activation of the LDL receptor gene (14, 24). Such activation would increase cellular cholesterol uptake from exogenous LDL. The requirement for a distinct ubiquitous factor for genes such as FPP synthase and HMG-CoA synthase, which are involved in intracellular sterol synthesis, provides the basis for possible independent regulation of sterol uptake and sterol synthesis. It is interesting to note that NF-Y (21) and Sp1 contain glutamine-rich activation domains and that the glutamine activation domains of Sp1 are required for activation of the LDL receptor promoter along with SREBP (24). It will be of interest to determine whether adipocyte genes that are activated by adipocyte differentiation and differentiation factor 1 (ADD-1), the rat homologue of SREBP (25, 26), also require NF-Y for normal function. Based on the current studies and on data not shown, we propose that NF-Y plays a novel and specific role in the regulation of key genes involved in lipid biosynthesis and that it functions by specific interactions with SREBP (ADD-1) or related proteins.

Acknowledgments—Anti-NF-YA monoclonal antibody and pNF-YA29 were generous gifts from Dr. R. Mantovani. Anti-CBF-A rabbit antiserum and anti-CBF-HSP were generous gifts from Drs. B. de Crombrugghe and B. Wu, respectively. We also thank Dr. D. Spear for helpful discussions and Bernard Lee for excellent technical assistance.

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doi: 10.1074/jbc.270.37.21445

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