Dependence of Rat Spot14 Promoter Activity on NF-Y Binding to the Inverted CCAAT-element at −100

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Electrophoretic mobility shift assay (EMSA) and in vitro transcription/translation show that NF-Y binds to the inverted CCAAT-element in the promoter of the rodent spot14 gene. The NF-Y-binding sequence has been shown to be responsible for basal activity in H4IE. Given the similar role found for the inverted CCAAT-element in the promoter of the FAS gene, NF-Y may have an important function in the control of lipogenesis.

Key words: spot14 promoter; fatty acid synthase; NF-Y; inverted CCAAT-element

The rat spot14 gene is regulated by tissue-specific, hormonal, developmental and nutritional factors. Although the function of the spot14 protein is still unknown, similarities in its regulation to that of other lipogenic enzymes suggest that it functions in lipid metabolism. For example, the transcription of spot14 and fatty acid synthase (FAS) are both up-regulated during postnatal development by insulin, a carbohydrate-rich diet and T3 (triiodothyronine), while spot14 is down-regulated in diabetes, during starvation and by PUFA (polyunsaturated fatty acid) and cAMP. MacDougald and Jump have identified a cis-acting element between −113 and −88 (B-region) consisting of an inverted CCAAT-element comparable to ICE in the FAS promoter. We have characterized two binding sites for the ubiquitously expressed transcription factor NF-Y (nuclear factor Y) in the FAS promoter: FIRE1 (FAS insulin-responsive element 1) at −509, and ICE (inverted CCAAT-element) at −100, which is essential for basal promoter activity and also has an important role in the regulation of the FAS promoter by cAMP, and all trans-retinoic acid. We show here by EMSA and transient transfection that NF-Y binds to the inverted CCAAT-element between −113 and −88 in the spot14 promoter, suggesting that this transcription factor may be instrumental in the regulation of the two lipogenic enzymes, FAS and spot14.

MacDougald and Jump have shown by in vitro DNAs footprinting of the rat spot14 promoter between −464 to −8 that it contained four protected regions: A (−63 to −48), B (−113 to −88), C (−244 to −227) and D (−310 to −286). The B-region containing an inverted CCAAT-element between −104 and −100 enhanced the initiation of transcription as measured by an in vitro transcription assay. EMSAs (electrophoretic mobility shift assays) have identified the binding of a transcription factor with high binding activity in a liver extract and low binding activity in a kidney extract. The fact that the inverted CCAAT-element in the FAS promoter bound NF-Y prompted us to determine whether or not NF-Y is the transcription factor binding to the inverted CCAAT-element located within the B-region of the spot14 promoter.

Competition experiments, using oligonucleotides corresponding to the B-region of the spot14 promoter and other known NF-Y binding sites in the rat FAS gene, FIRE1 and ICE, as well as defined NF-Y binding sites in other promoters confirmed that NF-Y in fact binds to the oligonucleotide corresponding to the sequence −115 to −87 of the spot14 promoter (data not shown). The CCAAT binding activity was found in nuclear extracts from HeLa, HepG2, MOLT-4 and differentiated 3T3-L1 adipocytes, underlining the fact that the protein(s) binding to the B-region are of the same ubiquitous nature as that known for NF-Y.

The results of the competition experiments strongly suggest that NF-Y was involved in binding to the B-region. This is not unexpected since the core sequence of the B-region shows a 9/11 match with the NF-Y consensus sequence 5’/G/CAGCCATC/GA/GGC/A-3’. We therefore incubated antisera against the A and B subunits of NF-Y with a rat liver
nuclear extract prior to EMSA (Fig. 1). The DNA/protein complex was found to be specifically supershifted by antisera against NF-YA and NF-YB (lanes 2 and 3). However, the non-specific rabbit IgG antiserum did not affect the specific DNA/protein complex (lane 4), indicating strongly that NF-Y is indeed involved in the binding of CCAATspot14.

Next, we wanted to test the direct interaction of NF-Y with CCAATspot14 by EMSA using in vitro synthesized NF-YA, NF-YB and CBF-C(NF-YC) (Fig. 2). When the individual in vitro translated NF-Y subunits were incubated with the CCAATspot14 oligonucleotide, a weak band could be observed, indicating the presence of NF-Y in the lysate (lanes 1–3). However, the binding signal was strongly enhanced in the presence of all three translation products, i.e. NF-YA, NF-YB and CBF-C (lane 4). This increased binding was specific, since the NF-Y-binding ICE oligonucleotide (CCAATICE) could compete for the binding of NF-Y (lane 5), whereas the unspecific competitor AP-1 (activating protein-1) did not (lane 6). Furthermore, the inclusion of the antiserum against NF-YB specifically supershifted the complex (lane 7). The increased binding signal caused by the in vitro translated NF-Y subunits (lane 4) corresponds to the band obtained with a rat liver nuclear extract (lane 9). Mantovani et al.14 have shown recently that the mutant form of NF-YA, NF-YAm29 in which three essential amino acids in the putative C-terminal helix necessary for DNA-binding are all replaced by alanine, was strongly affected in DNA binding but still able to interact with NF-YB. Using in vitro translated NF-YAm29 together with NF-YB and CBF-C, no binding could be observed (lane 8).

Transfection of rat hepatoma cell line H4IIE was carried out with two reporter plasmids, one corresponding to the wild-type spot14 promoter and the other carrying a mutation in the inverted CCAAT-element in which ATTGG was altered to ATTTA. Measurement of the luciferase activity of cells transfected with these plasmids showed a dramatic loss of activity with the reporter plasmid containing the mutated version of the spot14 inverted CCAAT-element. The 60% reduction in reporter activity of the mutated plasmid is shown in Fig. 3. The results of these transfection experiments confirm the functional role of the inverted CCAAT-element in the basal activation of the spot14 gene. This could explain the results of MacDougald and Jump,20 showing that the spot14 promoter fragment from −151 to −8 supports 2.5-fold higher transcription than the region between −87 to −8.

![Fig. 2. In Vitro Translated NF-YA, NF-YB and CBF-C(NF-YC) Bind together to the Inverted CCAAT-Element of the Spot14 Promoter.](image)

EMSA was performed with the labelled CCAATspot14 oligonucleotide in the presence of in vitro transcribed/translated NF-YA, NF-YB, CBF-C and NF-YAm29 as indicated above each lane. Competition experiments were carried out by using a 50-fold molar excess of each unlabelled competitor as indicated. B is the DNA/protein complex, and U is an unspecified DNA/protein complex.

**Fig. 1.** NF-Y Binds to the Inverted CCAAT-Element at -100 in the Spot14 Promoter.

The 32P-labelled double-stranded CCAATspot14 oligonucleotide (−115 to −87 (5′-CACCGTGGATGGCTCAAAA-CAAGGC-3′))21 was incubated with a rat liver nuclear extract together with antiserum against NF-YA or NF-YB as indicated in lanes 2 and 3. Rabbit IgG antiserum was used as a control in lane 4. B is the specific DNA/protein complex, while Bs is the supershifted complex with anti-NF-YA, and Bsa is the supershifted complex with anti-NF-YB.
Fig. 3. Basal Activity of the Spot14 Gene Promoter Depends on an Intact CCAAT-Element.

Fusion constructs in pGL3 (Promega, Southampton) containing either the region between −115 to +8 as in the wild type (WT) or the corresponding region mutated in the CCAAT-element (5′-CAACGCTGG GATTAA CTCAAAA-CAAGGC-3′ = MUT) were transfected into rat hepatoma cell line H4IIE as described.10 The relative luciferase activity for the WT promoter construct was set arbitrarily at 100%. The results shown were obtained from 6 independent transfections, each being performed in triplicate.

In conclusion, we have shown that NF-Y binds to the inverted CCAAT-element of the B-region between −115 and −87 in the promoter of the rat spot14 gene, the same location as that of the inverted CCAAT-element (ICE) in the rat FAS promoter.5 Deletion of ICE from the FAS promoter resulted in a 70% reduction in luciferase activity in HepG2 and HeLa cells.11 Prevention of NF-Y binding to the inverted CCAAT-element at −100 of the spot14 gene resulted in a 60% reduction in the basal reporter gene activity in H4IIE cells. Although we could not detect any changes in the NF-Y binding pattern after T3 or PUFA treatment of H4IIE cells (data not shown) and despite its abundance in different cell types, NF-Y may well prove to be the mediator for T3-specific signals.15 The data presented here corroborate the results,15 regarding the binding of NF-Y in the promoter of the spot14 gene. Given the fact that, in spite of its unknown function, spot14 is considered to be involved in lipogenesis, the ubiquitous transcription factor NF-Y may well play a role in the regulation of this process.

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References

