Transcriptional Stimulation by Hepatocyte Nuclear Factor-6

TARGET-SPECIFIC RECRUITMENT OF EITHER CREB-BINDING PROTEIN (CBP) or p300/CBP-ASSOCIATED FACTOR (p/CAF)*

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Vincent J. Lannoy‡, Annie Rodolosse§, Christophe E. Pierreux¶, Guy G. Rousseau, and Frédéric P. Lemaigre†

From the Hormone and Metabolic Research Unit, Université catholique de Louvain and Christian de Duve Institute of Cellular Pathology (ICP), Avenue Hippocrate 75, B-1200 Brussels, Belgium

Transcription factors of the ONECUT class, whose prototype is HNF-6, contain a single cut domain and a divergent homeodomain characterized by a phenylalanine at position 48 and a methionine at position 50. The cut domain is required for DNA binding. The homeodomain is required either for DNA binding or for transcriptional stimulation, depending on the target gene. Transcriptional stimulation by the homeodomain involves the F48M50 dyad. We investigate here how HNF-6 stimulates transcription. We identify transcriptionally active domains of HNF-6 that are conserved among members of the ONECUT class and show that the cut domain of HNF-6 participates to DNA binding and, via a LXXLL motif, to transcriptional stimulation. We also demonstrate that, on a target gene to which HNF-6 binds without requirement for the homeodomain, transcriptional stimulation involves an interaction of HNF-6 with the coactivator CREB-binding protein (CBP). This interaction depends both on the LXXLL motif of the cut domain and on the F48M50 dyad of the homeodomain. On a target gene for which the homeodomain is required for DNA binding, but not for transcriptional stimulation, HNF-6 interacts with the coactivator p300/CBP-associated factor but not with CBP. These data show that a transcription factor can act via different, sequence-specific, mechanisms that combine distinct modes of DNA binding with the use of different coactivators.

Cell differentiation and maintenance of the differentiated phenotype rely on the cell type-specific expression of genes. This expression is tightly controlled by transcription factors that display a restricted tissue distribution. The study of transcription factors has identified their protein domains involved in transcriptional activation or repression and in DNA binding and has led to classification of these factors on the basis of the structure of such domains. The liver-enriched transcription factors belong to six families (1). These are the CCAAT/enhancer-binding proteins (2) and the proline/acid-rich factors (3), which contain a leucine zipper; the hepatocyte nuclear factor (HNF)1 family of proteins, which contain a variant homeodomain (4–6); the HNF-3 family, which contains a forkhead domain (7, 8); the HNF-4 factors (9) and fetoprotein transcription factor/human B1-binding factor/CYP7A promoter-binding factor (10–12), which are steroid receptor-related and have a zinc-finger DNA-binding domain; and the ONECUT proteins HNF-6 and OC-2 (13–15).

Transfection studies (13, 16–20) have shown that, in the liver, HNF-6 controls transcription of the genes that code for HNF-3β and HNF-4, for plasma transport proteins (transferrin, α-fetoprotein), for coagulation factors (protein C), and for enzymes that regulate glucose metabolism (6-phosphofructo-2-kinase) or steroid metabolism (Cyp2C12). A number of other genes expressed in the liver contain HNF-6 binding sites, but their actual control by HNF-6 has yet to be demonstrated (16). HNF-6 is also a mediator of growth hormone action (18) and a modulator of glucocorticoid action on the 6-phosphofructo-2-kinase and phosphoenolpyruvate carboxylkinase genes in the liver (20). During embryonic life, HNF-6 is expressed starting at the onset of pancreas development (19, 21), suggesting that it plays a role in this process. This has been confirmed by investigations on hnf6–/– mice (22).

The ONECUT proteins contain a bipartite DNA-binding domain composed of a single cut domain and a divergent homeodomain. Homeodomains are 60 residues long and are organized into three α-helices. Within the DNA recognition helix, amino acid 48 of the homeodomain is part of the hydrophobic core, and amino acid 50 is essential for sequence-specific DNA binding (23, 24). In the ONECUT proteins, residue 48 is a phenylalanine, not a tryptophan as in all of the other homeo-proteins; residue 50 is a methionine, an amino acid never found at this position in the other homeodomains. The F48M50 dyad is therefore characteristic of the homeodomain of ONECUT proteins. Our studies on the DNA binding properties of HNF-6 (14) showed that the cut domain is required for binding to all HNF-6 sites, while the homeodomain is required for binding to only a subset of sites. Mutation of the F48M50 dyad into tryp-

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‡ Recipient of a fellowship from the Fonds pour la Formation à l’Industrie et l’Agriculture (Belgium).
§ Present address: Laboratory of Neurobiology, University of Zaragoza, 50013 Zaragoza, Spain.
¶ Present address: Developmental Signalling Laboratory, Imperial Cancer Research Fund, London WC2A 3FX, United Kingdom.
† Present address: Hormone and Metabolic Research Unit, Université catholique de Louvain and Christian de Duve Institute of Cellular Pathology (ICP), Avenue Hippocrate 75, B-1200 Brussels, Belgium.

1 The abbreviations used are: HNF, hepatocyte nuclear factor; TTR, transthyretin; CBP, CREB-binding protein; p/CAF, p300/CBP-associated factor; SRC-1, steroid receptor coactivator-1; RAC-3, receptor-associated coactivator-3; EMSA, electrophoretic mobility shift assay; STP, serine/threonine/proline-rich; GST, glutathione S-transferase.
tophan and histidine (W48H50), which converts the divergent homeodomain into a classical homeodomain, does not affect DNA binding. However, this mutation reduces the transcriptional stimulation of those target genes to which HNF-6 binds without requirement for the homeodomain. These observations indicate that the homeodomain of HNF-6 has a dual role; it is involved either in DNA binding or in transcriptional stimulation, depending on the target gene.

Based on sequence alignments of their cut domains and of their homeodomains, the ONECUT proteins, which have been found in mammals and in Caenorhabditis elegans, appear as a separate class of cut homeoproteins (14). A further comparison of their amino acid sequence (15) revealed the presence of conserved regions outside the cut domain and the homeodomain, namely a serine/threonine/proline-rich region, which we call the STP box, and an internal serine-rich region. In addition, the two known mammalian ONECUT proteins, HNF-6 and OC-2, contain a polyhistidine tract located downstream of the STP box.

How the HNF-6 homeodomain stimulates transcription and how the HNF-6 regions conserved among the ONECUT proteins are involved in transcriptional control is unknown. We characterize here the function of the STP box, of the C-terminal serine-rich region and of the polyhistidine tract of HNF-6. Moreover, we show that HNF-6 recruits either the coactivator CREB-binding protein (CBP) or p300/CBP-associated factor (pCAF), depending on the type of target sequence bound by HNF-6. The interaction of CBP with HNF-6 involves the serine-rich region and of the polyhistidine tract of HNF-6.

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**Experimental Procedures**

**Plasmid Constructions**—pECE-HNF6a and pECE-HNF6a/F48W+ M50H have been described (13, 14). pECE-HNF6a/Ser codes for amino acids 1–451 of HNF-6a and was obtained by cloning the EcoNII/XbaI fragment derived from pECE-HNF6a digested by EcoNII/XbaI. pECE-HNF6a/STP (amino acids 1–98 and 123–465 of HNF-6a) and pECE-HNF6a/2PH (amino acids 1–122 and 139–465 of HNF-6a) were generated by subcloning the polymerase chain reaction products obtained with the following primers: 5′-CCGGAGATCTGAATCGGTTCT-3′ (BeSTP), 5′-CCGGAGATCTGGAGATCTAT-3′ (STP), 5′-CCGGAGATCTGCCGCGTGG-3′ (BePH), 5′-CCGGAGATCTCAGCACTCTTGAC-3′ (STP), 5′-CCGGATCTCAGCACTCTTGAC-3′ (STP) (V0). The transformation of the cut domain and the STP box.

**RESULTS**

Identification of Domains of HNF-6 That Control Transcription—The sequence alignment of the proteins of the ONECUT class, to which HNF-6 belongs, revealed the presence of three conserved regions outside the cut domain and the homeodomains. These are a 24-residue-long serine/threonine/proline-rich region (STP box) corresponding to amino acids 99–122 of HNF-6, a polyhistidine tract (amino acids 129–138), and a C-terminal serine-rich region (amino acids 449–465) (15). To identify the role of these domains, we constructed expression vectors for HNF-6 mutants devoid of these domains and tested their activity on two control reporter constructs in transfected rat hepatoma FTO-2B cells. One reporter contains the cut domain alone, and the other contains the cut domain under the control of a TATA box and six copies of the HNF-6 site found in the hnf3β gene. The other reporter is identical, except that the HNF-6-3 sites are derived from the transhyretin (ttr) gene. These two reporters were chosen because we had found (14) that binding of HNF-6 to the HNF-3β-type reporter requires only the cut domain, while binding to the HNF-6 reporter requires both the cut domain and the TTR-type reporter.
and the homeodomain. Moreover, the homeodomain is required for transcriptional stimulation via the HNF-3β site but not via the TTR site (14).

We first verified by EMSA that the various HNF-6α mutants bound at least as well as wild-type HNF-6α to the HNF-6 binding sites derived from the trt and hnf3β genes. This was the case (Fig. 1A). We then tested their transcriptional activity. As shown in Fig. 1B, deletion of the C-terminal serine-rich domain (HNF-6αΔSer) or of the polyhistidine tract (HNF-6αΔPH) increased slightly the transcriptional activity of HNF-6α on both types of target sequences, suggesting that these regions are inhibitory. In contrast, deletion of the STP box (HNF-6αΔSTP) decreased by about half the activity of HNF-6α on the two reporter plasmids. This effect was more severe on the TTR-type reporter (75% of reduction) than on the HNF-3β-type reporter (50% of reduction). The reduced transcriptional activity of the L350A mutant was not a consequence of decreased DNA binding, as could have been suspected from the known activity of the L350A mutant on the two reporter plasmids. This effect was more severe on the TTR-type reporter (75% of reduction) than on the HNF-3β-type reporter (50% of reduction). The reduced transcriptional activity of the L350A mutant was not a consequence of decreased DNA binding, as could have been suspected from the known activity of the L350A mutant on the TTR- or HNF-3β-type reporter constructs. This motif has been identified as being involved in transcription factor/coactivator interactions (25, 26). We therefore determined whether the LSDLL sequence in the cut domain of HNF-6 plays a role in transcriptional activation. To do so, we constructed an expression vector coding for HNF6αL350A in which the LSDLL sequence has been mutated into LSDAL (Fig. 1B). This mutation abolishes the functionality of the LXXLL motif in terms of interaction with proteins (26). The activity of the L350A mutant on the TTR- or HNF-3β-type reporter constructs was tested by transfection in FTO-2B cells. As shown in Fig. 1B, mutation of the LSDLL sequence present in the cut domain reduced the transcriptional activity of HNF-6α on the two reporter plasmids. This effect was more severe on the HNF-3β-type reporter (75% of reduction) than on the TTR-type reporter (50% of reduction). The reduced transcriptional activity of the L350A mutant was not a consequence of decreased DNA binding, as could have been suspected from the known activity of the cut domain in binding to the TTR and HNF-3β sites (14). Indeed, EMSA showed that HNF-6α and HNF-6αL350A bound equally well to the two probes tested (Fig. 1C). We conclude that the cut domain of HNF-6 is required not only for DNA binding, but also for transcriptional stimulation via the two types of HNF-6 binding sites, and that it is therefore bifunctional.
CBP Is a Target-specific Coactivator of HNF-6—LXLL motifs are known to interact with CBP. This protein, which is present in limiting amounts, does not bind DNA but instead acts as a bridge between transcription factors and the transcriptional machinery (25, 27–29). To test the hypothesis that HNF-6 uses CBP as a coactivator, we transfected FTO-2B cells with the HNF-6α or HNF-6αL350A expression vector in the presence of increasing amounts of a CBP expression vector and with the HNF-3β-type reporter construct. Consistent with our hypothesis, CBP increased the activity of wild-type HNF-6α in a dose-dependent manner (Fig. 2A). Coactivation by CBP was abolished when transcription of the reporter was stimulated by HNF-6αL350A instead of HNF-6α (Fig. 2B). This indicated that the LSDLL sequence of HNF-6 is required for coactivation by CBP and suggested that CBP interacts with HNF-6α via this sequence. To investigate this hypothesis, we performed in vitro GST pull-down experiments (Fig. 3A). Bacterially expressed fusion proteins consisting of portions of CBP linked to GST were immobilized on glutathione-Sepharose beads and tested for their ability to retain radioactively labeled recombinant HNF-6α. These experiments showed that the N-terminal third (amino acids 1–1098) of CBP specifically interacts with HNF-6α. In contrast, this fragment of CBP did not interact with HNF-6αL350A (Fig. 3B). This indicates that the LSDLL sequence of HNF-6 is required for coactivation by CBP and suggested that CBP interacts with HNF-6α via this sequence.

Our earlier experiments (14) had shown that stimulation of the HNF-3β-type reporter by HNF-6 requires an intact F48M50 dyad in the HNF-6 homeodomain. We therefore verified if the F48M50 dyad is involved in coactivation with CBP. This was done by transfecting an HNF-6α mutant in which the F48M50 dyad has been changed to W48H50 (14). This not only reduced transcriptional stimulation, consistent with earlier work (14), but also abolished coactivation by CBP (Fig. 2C). These effects of the F48W1M50H mutation were not due to decreased HNF-6 binding to the reporter gene, since we have shown earlier that this mutation does not reduce DNA binding (14). This is consistent with the fact that the homeodomain is not involved in HNF-6 binding to the HNF-3β-type reporter (14). To test whether the F48M50 dyad is also required for the interaction of HNF-6 with CBP, we repeated the GST pull-down of CBP-(1–1098) with the HNF-6αF48W1M50H mutant. As shown in Fig. 3C, the amount of HNF-6αCBP complex relative to the input was 5 times less with the F48W1M50H mutant than with wild-type HNF-6α. This indicates that HNF-6αF48W1M50H bound much less well to CBP than wild-type HNF-6α. We conclude that, on an HNF-3β-type of target, CBP is a coactivator of HNF-6 and that coactivation occurs via an interaction of HNF-6 with CBP that involves the LSDLL sequence of the cut domain and the F48M50 dyad of the homeodomain. The mutation of the LSDLL sequence in the HNF-6 cut domain reduced transcriptional stimulation not only of the HNF-3β-type reporter but also of the TTR-type reporter (Fig. 2).
interaction of the latter two domains with p/CAF. Finally, deletion of amino acids 139–347 generated a mutant that was not expressed (data not shown). We conclude from this set of experiments that, on a TTR type of target, p/CAF is a coactivator of HNF-6 and interacts with it in an LSDLL-independent way.

**DISCUSSION**

The data presented here show that HNF-6 displays two modes of transcriptional stimulation, which are determined by the way in which it interacts with DNA, this in turn depending on the sequence of target DNA. On one type of target, whose prototype is the HNF-6 binding sequence present in the hnf3β promoter, DNA binding of HNF-6 only requires its cut domain. In this case, transcriptional stimulation involves the LSDLL sequence of the cut domain and the F48M50 dyad of the homeodomain; HNF-6 interacts with CBP and uses it as a coactivator. On another type of target, whose prototype is the HNF-6 binding sequence present in the ttr gene promoter, DNA binding of HNF-6 requires both the cut domain and the homeodomain. In this case, transcriptional stimulation involves the cut domain but not the homeodomain. On this type of target, HNF-6 does not interact with CBP but rather with p/CAF. Thus, HNF-6 recruits either CBP or p/CAF in a target-specific way. Coactivation of a TTR-type target by p/CAF does not depend on the HNF-6 LSDLL sequence, although the integrity of the latter is required for full activation of such a target.

Work by others has provided examples of a single DNA-binding protein recruiting different transcriptional activators, depending on the context. However, none of them uses the same discriminatory mechanism as HNF-6. The T cell factor LEF-1, which contains a high mobility group DNA-binding domain but lacks an activation domain, needs a partner to stimulate transcription. This partner is β-catenin on the c-fos promoter or ALY (ally of LEF-1) on the TCRα enhancer. In contrast to HNF-6, the LEF-1 binding sequence is the same in the two targets, and the mechanism of partner discrimination probably depends on the interaction of LEF-1 with different factors that bind in the vicinity (30). For nuclear hormone receptors, the sequence of the DNA target can be mechanistically discriminatory, as is the case for HNF-6. Here, however, binding of the same receptor to different sequences determines whether the hormonal ligand will trigger transcriptional stimulation via a coactivator or transcriptional inhibition via a corepressor. Another difference with HNF-6 is that nuclear receptors have a single DNA-binding domain, and it is via their homo- or heterodimerization that the sequence-dependent transcriptional specificity is achieved (31). For HNF-6, it is thanks to the combinatorial use of two separate, bifunctional, domains that distinct target sequences impose distinct modes of DNA binding, which, in turn, determine different mechanisms of transcriptional stimulation.

The LSDLL sequence in HNF-6 is required for transcriptional stimulation of the two types of target sequences studied here. On the HNF-3β-type target, our data show that the LSDLL sequence is required for the interaction with CBP and that this interaction is involved in transcriptional stimulation. On the TTR-type target, p/CAF interacts with HNF-6, but this does not require the HNF-6 LSDLL sequence. This suggests that, on a TTR-type target, HNF-6 may interact with p/CAF and with an additional coactivator that would bind to the LSDLL sequence.

In the present work, we demonstrate that the cut domain of HNF-6 is involved not only in DNA binding but also in transcriptional stimulation. The cut domain occurs in three copies in the homeoproteins of the Cux class, which includes the *Drosophila* cut protein and its mammalian homologs (14). To
our knowledge, there is no evidence that the cut domain of these proteins has a function(s) other than DNA binding. One of the three cut domains of the CUX class proteins contains a VSDLL sequence. Such a sequence in the receptor interacting protein-140 retains the ability to bind CBP and is therefore compatible with the LXXLL motif (26). If the CUX class proteins recruit CBP, it is unlikely that they do so by the same mechanism as HNF-6. Indeed, we have shown here that recruitment of CBP by HNF-6 involves not only the cut domain but also the homeodomain and that changing the F48M50 dyad of HNF-6 to the W48H50 dyad of the CUX class proteins prevents coactivation by CBP. This may explain the evolutionary conservation of these critical residues within the ONECUT class. Interestingly, recruitment of p300, a homolog of CBP, by the homeoprotein NK-4 has been reported recently (32). In contrast to HNF-6, which binds the N-terminal moiety of CBP, NK-4 binds the C-terminal moiety of p300, and this involves mainly the N-terminal portion (activation domain) of NK-4.

CBP serves as a docking platform for several nuclear proteins that are end points of transduction cascades triggered by cell surface signaling (33). Since CBP is present in rate-limiting amounts in cells, its concomitant recruitment by different transcription factors on the same gene can promote synergistic stimulation of transcription, whereas competitive recruitment on different promoters may lead to antagonistic interactions (34). This may be relevant to the functioning of the liver-specific transcription factors. Indeed, D-binding protein (35), a member of a network of liver-specific transcription factors that is regulated by extracellular signals such as insulin (39) and growth hormone (40). Our identification of HNF-6 as a partner of CBP broadens our understanding of this integrative mechanism. First, this hints at a possible modulation of HNF-6 activity by extracellular signals. Second, insofar as HNF-6 is a tissue-restricted transcription factor that recruits CBP to only a subset of its target genes, our model provides a new mechanism for the tissue-specific and gene-specific transcriptional effects of such signals.

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