Nuclear Factor I Regulates Expression of the Gene for Phosphoenolpyruvate Carboxykinase (GTP)*

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Deborah R. Crawford‡§, Patrick Leahy‡§, Ching Y. Hu, Ali Chaudhry§, Richard Gronostajski, Gregory Grossman, Jason Woods, Parvin Hakimi, William J. Roesler**, and Richard W. Hanson‡‡

From the Department of Biochemistry, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106-4935, the Department of Cancer Biology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio 44106, and the **Department of Biochemistry, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5E5, Canada

Nuclear factor-I (NFI) binds to the phosphoenolpyruvate carboxykinase (GTP) (PEPCK) gene promoter immediately 5' to the cAMP regulatory element (CRE). This suggests an interaction between NFI and factors that bind the CRE. Of the four NFI isoforms expressed in mammalian tissues, NFI-A and -B stimulate basal transcription from the PEPCK gene promoter in HepG2 cells, while NFI-C and -X are slightly inhibitory. All four NFI isoforms abrogate the 20-fold protein kinase Ac (PKAc)-mediated induction of transcription from the PEPCK gene promoter. Normal PKAc-mediated induction was noted when the CRE was moved 10 base pairs 3' of its original location. However if the CRE was moved 5 base pairs 3', placing it out of phase with the other elements in the promoter, or moved 5' to -285 (the P3(1) site in the promoter), some PKA-mediated stimulation was lost. The NFI-C isoform effectively inhibited PKAc induction regardless of the relative positions of the CRE and the NFI binding sites. NFI-C also abrogated cAMP regulatory element-binding protein (CREB)-induced activity of wild type and mutant PEPCK promoters. There was some cooperativity in the binding of CREB and NFI to their respective binding sites but this did not appear to be physiologically important.

Phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) (PEPCK) is a key enzyme in hepatic and renal gluconeogenesis, whose activity is tightly regulated at the transcriptional level. A number of transcription factors have been identified that interact with the PEPCK gene promoter to control expression (1). Among these is the NFI family of site-specific DNA-binding proteins (2). The consensus sequence, TTGCC(N)3-GCCAA, to which NFI binds (2–4) is present on the PEPCK promoter (P1 site) immediately adjacent to a cAMP response element. The latter is important for both basal and cAMP-induced transcription (5) (Fig. 1). Purified NFI binds the P1 site of the PEPCK gene promoter (6). Since all four NFI genes are expressed at high levels in liver (7, 8) where PEPCK is also strongly expressed, it seemed likely that NFI would play a role in the regulation of PEPCK expression.

The proximity of the P1 and CRE sites in the PEPCK gene promoter raises the possibility of interactions between the NFI isoforms and protein(s) that bind the CRE. NFI isoforms have been implicated as co-factors necessary for the full transcriptional response of genes to various stimuli (9). NFI is also involved in glucocorticoid receptor-mediated transcriptional response of the gene for mouse mammary tumor virus (10) and aspartate aminotransferase (11). In addition, NFI has been suggested as a negative modulator of transcription of the gene for L-type pyruvate kinase (12). Footprint analysis of the PEPCK gene promoter has shown that both the P1 and CRE sites on the endogenous PEPCK gene were occupied under conditions of basal gene expression (13, 14). Christ et al. (15) reported that an oligonucleotide containing the NFI binding site from the rat PEPCK gene promoter bound greater amounts of protein from rat liver nuclear extracts when the site was placed in context next to the CRE, suggesting that the CRE is involved in recruiting proteins to the NFI binding site. In the present study, we report the effects of individual NFI isoforms on basal and PKAc-induced transcription from the PEPCK gene promoter and examine the issue of position sensitivity of the P1 and CRE sites.

**EXPERIMENTAL PROCEDURES**

*These authors contributed equally to the work contained in this manuscript and may be considered co-first authors.

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¶ Present address: Dept. of Animal Sciences, Oregon State University, Corvallis, Oregon 97331.

**To whom correspondence should be addressed: Dept. of Biochemistry, Case Western Reserve University School of Medicine, Cleveland, OH 44106-4935. Tel.: 216-368-5302; Fax: 216-368-4544; E-mail rwh@po.cwru.edu.

Materials

**1**H-Chloramphenicol (30–60 g/mol) and **3**P-dCTP (3000 Ci/mol) were purchased from NEN Life Science Products. 8-Bromo-cAMP and butyryl coenzyme A were purchased from Sigma. HepG2 cells were originally purchased from ATCC (Rockville, MD). The expression vector for catalytic subunit of protein kinase A was a gift from Dr. Masa-Aki Muramatsu. DMEM/F-12 cell culture medium and fetal calf serum were from Life Technologies, Inc. All other reagents used in this study were of the highest quality obtainable. CREB was partially purified from a crude lysate of Escherichia coli; the CREB expression vector was a kind gift of Dr. Marc R. Montminy. All expression vectors for the NFI proteins were the same as those used in a earlier study (8).

**Methods**

Construction of Chimeric Genes—The details of the construction of the chimeric PEPCK-CAT genes used in the transfections experiments (CRE2CAT, CRE3CAT, and CRE2+3CAT) were outlined previously.

The abbreviations used are: PEPCK, phosphoenolpyruvate carboxykinase; GRE, glucocorticoid regulatory element; CBP, CREB-binding protein; NFI, nuclear factor-I; CRE, cAMP regulatory element; PKA, protein kinase A; PKAc, protein kinase A catalytic subunit; CREB, cAMP regulatory element-binding protein; URE, upstream regulatory element; DMEM, Dulbecco's minimal essential medium; CAT, chloramphenicol acetyltransferase.
Nuclear Factor I Regulates PEPCK Gene Transcription

(16). Block mutations were introduced into the PEPCK gene promoter as described by Liu et al. (5). C0.5CAT and C1.0CAT contained the following modified promoter regions respectively: CRE0.5CAT, −100-GCCGGCCAGCGGTCAAGAGTCCAGCTGTCAG; CRE1.0CAT, −100-GCCGGCCAGCGGTCAAGAGTCCAGCTGTCAG. The effects of the four isoforms of NFI on cAMP-induced transcription from the PEPCK gene promoter (Fig. 2). NFI-A and NFI-B stimulated basal transcription 6- and 4-fold, respectively, while NFI-C and NFI-X had no effect or caused a slight inhibition. All four NFI isoforms abrogated the normally robust (20–40-fold) induction of transcription from the PEPCK gene promoter caused by PKAc. NFI-C and NFI-X totally inhibited induction of transcription to levels below basal, while NFI-A and NFI-B reduced transcription to approximately the levels noted when the these two isoforms were co-transfected in the absence of PKAc. The effect of NFI on PKAc induced transcription from the PEPCK gene promoter was not caused by NFI inhibition of the expression of CMV-PKAc gene, since the same pattern of effect of NFI-C on transcription from the PEPCK promoter was noted when 8-bromo-cAMP was added directly to the medium (Fig. 2, Inset). Thus, NFI isoforms have a dominant negative effect on cAMP induction of transcription from the PEPCK gene promoter and NFI can set the overall level of expression despite the presence of cAMP, a powerful stimulus of PEPCK gene transcription.

The functional significance of the difference in the effects of the various isoforms of NFI on basal transcription from the PEPCK gene promoter is not clear. RNA for each of the four vertebrate genes for NFI are differentially spliced to yield 18 distinct isoforms, many of which exhibit a unique species distribution (2, 4, 20). Chaudhry et al. (8) have studied the pattern of expression of the isoforms of NFI and found that all four of the forms are highly expressed in the liver, whereas NFI-C is most highly expressed in the kidney. The pattern of embryonic expression of the various isoforms of NFI is more complex. NFI-A is expressed earliest during fetal development of the heart (at 9 days of fetal life). In the developing liver, where PEPCK gene expression is greatest after birth, the levels of NFI-C and NFI-X are highest during fetal life; NFI-A and NFI-B are not detected in the liver in utero (8). Both NFI-C and NFI-X strongly inhibit cAMP induction of PEPCK gene transcription, which may explain why Patel et al. (19) observed

RESULTS AND DISCUSSION

An important role for NFI in the control of PEPCK gene transcription can be inferred from the experiments of Patel et al. (19). They demonstrated that a mutation of the NFI binding site in a PEPCK transgene promoter lead to increased levels of basal transcription from the promoter in the livers of transgenic mice. In utero abnormalities such as premature expression of the transgene and loss of the normal cAMP-mediated capability for stimulation of expression were also observed. In another study, mice carrying a mutated NFI site in a PEPCK transgene promoter showed a marked increase in the basal hepatic expression of the transgene in response to hypoxia.2 Taken together, these findings suggest that NFI plays a key role in maintaining a low level of basal expression of the PEPCK gene. A similar role for NFI has been proposed for the liver-type pyruvate kinase (12). NFI may thus be critical for maintaining low levels of PEPCK to ensure that there is control of glucose overproduction by the liver.

To determine the effect of NFI on PEPCK gene transcription, we tested the ability of the four isoforms of NFI to alter the basal and cAMP-stimulated transcription from the PEPCK gene promoter (~490 to +73) linked to the CAT structural gene in HepG2 cells. The various isoforms of NFI had different effects on transcription from the PEPCK gene promoter (Fig. 2). NFI-A and NFI-B stimulated basal transcription 6- and 4-fold, respectively, while NFI-C and NFI-X had no effect or caused a slight inhibition. All four NFI isoforms abrogated the normally robust (20–40-fold) induction of transcription from the PEPCK gene promoter caused by PKAc. NFI-C and NFI-X totally inhibited induction of transcription to levels below basal, while NFI-A and NFI-B reduced transcription to approximately the levels noted when the these two isoforms were co-transfected in the absence of PKAc. The effect of NFI on PKAc induced transcription from the PEPCK gene promoter was not caused by NFI inhibition of the expression of CMV-PKAc gene, since the same pattern of effect of NFI-C on transcription from the PEPCK promoter was noted when 8-bromo-cAMP was added directly to the medium (Fig. 2, Inset). Thus, NFI isoforms have a dominant negative effect on cAMP induction of transcription from the PEPCK gene promoter and NFI can set the overall level of expression despite the presence of cAMP, a powerful stimulus of PEPCK gene transcription.

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2 T. Riley, S. Kalhan, and R. W. Hanson, unpublished results.
premature expression of the gene for PEPCK in the livers of mice with a transgene in which the NFI binding site had been deleted. However, the physiological effects of the interactions of the various forms of NFI remain to be elucidated.

A number of other genes have promoters in which the NFI binding site and a CRE or a glucocorticoid regulatory element (GRE) are juxtaposed. The NFI site and the CRE in the human neurotropic JC virus early promoter have been shown to function independently of each other in controlling transcription in a tissue-specific manner (21). In addition, a close association between NFI and AP-1 proteins (Fos and Jun) has been demonstrated for the JC virus early promoter (22). The promoters of the genes for cytosolic form of aspartate aminotransferase (11) and for the long terminal repeat of the mouse mammary tumor virus (23) have glucocorticoid regulatory elements in close proximity to an NFI binding domain. A mutation in the NFI site of the aspartate aminotransferase gene promoter markedly decreases the level of basal gene transcription but permits glucocorticoid induction of transcription from the promoter in HepG2 cells. There is also evidence that the NFI site is required for PEPCK gene transcription in hepatoma cells (24). In all of the above studies the differential effect of the various isoforms of NFI on the transcription of the specific gene of interest was not determined so that functional variations of the isoforms of NFI on gene expression noted with the PEPCK gene promoter could be a more general phenomenon.

To explore whether any cooperative-type interaction existed between NFI-C and CREB, we carried out an electrophoretic gel mobility experiment in which a 63-base pair oligonucleotide, containing both CRE and NFI sites, was used as a probe to study the relative binding of purified CREB and NFI-C (Fig. 3). Lane 5 shows the bands shifted by a reaction which had three times the concentration of NFI relative to a standard concentration of CREB. The formation of the ternary complex should be limited by the protein in shortest supply. Therefore, we expected to see an increase in the band corresponding to NFI and no change in the bands for CREB or the band representing the ternary complex (when compared with lane 6). Clearly, the band representing the ternary complex was also increased in the presence of an increased concentration of NFI-C, suggesting that NFI recruited extra CREB, which bound to the DNA. A reciprocal effect may be inferred from the results shown in lane 4 where increased amounts of CREB in the reaction led not only to an increase in the CREB-shift but also to an increase in the band representing the ternary complex (compare lane 4 with lane 6). As a control, we demonstrated that the individual and ternary complex may be effectively removed by competition with unlabeled oligonucleotide corresponding to the CRE and NFI sites, respectively.
an expression vector for CREB produced uniform amounts of induction from the 490CAT, CRE0.5CAT, and CRE1.0CAT. NFI-C blocked the induction of transcription by CREB to the same degree (data not shown) with all of the above constructs, suggesting that proximity of the CRE and NFI sites is not important for function and that direct interaction between CREB and NFI is unlikely.

The results of these studies are surprising since they indicate that there is no position sensitivity associated with the proximity of location of the CRE and NFI binding sites on the PEPCK gene promoter. The mouse mammary tumor virus long terminal repeat (23) and the promoter for the estrogen-inducible vitellogenin gene (26) both contain an NFI binding site and mutations in this site alters steroid induction of gene transcription. Allgood et al. (10) have demonstrated, using an artificial promoter containing GRE and NFI sites, that there is no strict position or orientation requirements for cooperation between the NFI and GRE in glucocorticoid induction of transcription from the promoter. Since the NFI site in the PEPCK gene promoter is maintained in its original position in our experiments, it is probable that NFI either interacts with the TATA box-binding proteins or with a protein important in integrating the effects transcriptional control proteins, such as CBP/p300; the latter is involved in the control of transcription from the PEPCK promoter.3 In this regard, NFI is similar to CBP/p300; the latter is involved in the control of transcription from the PEPCK promoter.

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3 P. Leahy and R. W. Hanson, unpublished data.