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The C-terminal domain of the Nuclear Factor I-B2 isoform is glycosylated and transactivates the WAP gene in the JEG-3 cells

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Abstract

The transcription factor nuclear factor I (NFI) has been shown previously both *in vivo* and *in vitro* to be involved in the cooperative regulation of whey acidic protein (WAP) gene transcription along with the glucocorticoid receptor and STAT5. In addition, one of the specific NFI isoforms, NFI-B2, was demonstrated in transient co-transfection experiments in JEG cells, which lack endogenous NF-I, to be preferentially involved in the cooperative regulation of WAP gene expression. A comparison of the DNA-binding specificities of the different NFI isoforms only partially explained their differential ability to activate the WAP gene transcription. Here, we analyzed the transactivation regions of two NFI isoforms by making chimeric proteins between the NFI-A and B isoforms. Though, their DNA- binding specificities were not altered as compared to the corresponding wild type transcription factors, the C-terminal region of the NFI-B isoform was shown to preferentially activate WAP gene transcription in cooperation with GR and STAT5 in transient co-transfection assays in JEG-3 cells. Furthermore, determination of serine and threonine–specific glycosylation (*O*-linked *N*- acetylglucosamine) of the C-terminus of the NFI-B isoform suggested that the secondary modification by O-GlcNAc might play a role in the cooperative regulation of WAP gene transcription by NFI-B2 and STAT5.

Keywords

nuclear factor-1(NFI); whey acidic protein (WAP); glucocorticoid receptor (GR); signal trnaducer of activator of transcription 5(STAT5); composite response element (CoRE); *O*-linked *N*-acetylglucosamine (O-GlcNAc)

Introduction

The study of milk protein gene expression is an attractive system for examining hormonal and developmental regulation of gene expression, and furthermore, has provided insight into mammary gland-specific control of transcription [1]. Studies from several different laboratories have suggested that the spatially and temporally restricted milk protein gene expression patterns result from combinatorial interactions at the protein-protein and protein-DNA level of members of several families of commonly expressed transcription factors [2].

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The whey acidic protein (WAP), the major whey protein in rodents, is exclusively expressed at high levels in the mammary gland during late pregnancy and lactation, and has been employed as a model system to study the transcriptional regulation of milk protein gene expression. A distal region of the rat WAP gene, containing a composite response element (CoRE), which confers mammary gland-specific and hormonally-regulated expression, was identified previously in our laboratory [3,4]. In addition, the function of this CoRE was determined by the cooperative interactions among three transcription factors; a specific NFI isoform, the glucocorticoid receptor (GR) and STAT5A [2].

Individual NFI isoforms have been suggested to contribute to different stages of mammary gland development, in particular to gene expression in the terminally differentiated alveolar epithelial cells in late pregnancy and lactation as well as during tissue remodeling during involution. For example, NFI has been implicated in regulating the transcription of milk protein genes such as WAP, α-lactalbumin and β-lactoglobulin [5], as well as the lactation-associated transcription of β1,4-galactosyltransferase (β4-GT) and carboxylester lipase (CEL) genes [6, 7]. Furthermore, a mammary cell-specific enhancer in the mouse mammary tumor virus (MMTV) promoter includes an active NFI binding site [8]. In addition, the involution-associated transcription of the TRPM-2/clusterin gene has been shown to be controlled by a unique *N*-glycosylated 74 kDa NFI-C isoform [9].

NFI transcription factors can both activate and repress gene expression [10-12]. Furthermore, the same NFI isoforms can either activate or repress expression from the same promoter in a cell-type dependent manner. Transient transfection studies have shown that the NFI transcription factors are modular, with N-terminal domains mediating DNA binding and dimerization, and C-terminal domains mediating transactivation and repression [13-15]. The individual cell- and tissue-specific isoforms are the result of differential splicing of four NFI genes (A, B, C and X). NFI family members are highly homologous in their amino-terminal DNA binding and dimerization domains but are divergent in the carboxy-terminal transactivation-repression domains. Further diversity of the transactivation domain is accomplished by alternative splicing that creates regions of variable proline richness. The significance of the proline rich areas are not well understood [5].

N-Acetylglucosamine (*O*-GlcNAc) is a sugar residue that is used as a frequent post-translational modification of nuclear and cytoplasmic proteins. One molecule of GlcNAc is linked as a single monosaccharide to the hydroxyl group of serines or threonines and is not further elongated. Modification by O-GlcNAc is highly dynamic, and can give rise to functionally distinct protein subsets [16,17]. This reversible post-translational modification is present in a variety of proteins, including numerous chromatin-associated proteins and several transcription factors [18-20]. The ubiquitious transcription factors SP1, members of the AP-1 family, the estrogen receptor (ER), Pax-6, and c-Myc all carry O-GlcNAc residues [20]. A subset of the nuclear RNA polymerase II itself is also O-GlcNAc-modified at the carboxy-terminal moiety (CTD) of the largest subunit [21]. O-GlcNAcylation is highly dynamic, with rapid cycling in response to cellular signals or cellular stages. Although the function of O-GlcNAc has not been precisely determined, recent data suggest it can influence a wide variety of key cellular events, including gene transcription, insulin signaling, glucose metabolism, and cell cycle progression [16,17]. Gewinner et al. (2004) have shown that the glycosylated form of STAT5 binds with the coactivator of transcription CBP, which is very important for STAT5-mediated gene transcription [22].

In the present study, we compared the transactivation domains of two specific NFI isoforms expressed in the mammary gland, which are associated with WAP gene transcription. The chimeric protein (N-terminal of NFI-A4 and C-terminal of NFI-B2) with the C-terminal of NFI-B2, but not the C-terminal of NFI-A4 demonstrated cooperativity with GR and STAT5

in the hormonal induction of WAP gene transcription in JEG cells. Preliminary gel shift and co-immunoprecipitation assays suggest the O-GlcNAc modification of the C-terminus of NFI-B2 might play an important role in interaction of this specific NFI isoform with the STAT5 in WAP gene transcription.

Material and Methods

Plasmid construction

pWAPtk-luciferase, STAT5A, GR, prolactin receptor (PrlR) and the NFI constructs have been described previously [2]. To make the chimeric constructs (pCHNFI N-terA-C-terB and pCHNFI N-terB-CterA) between the NFI-A4 and NFI-B2, the pCHNFI-A4 and B2 were digested with *EcoN*I and *Not*I and the N-terminal fragment of A ligated with C-terminal fragment of B and vise versa. All the constructs were confirmed by sequencing.

Cell culture and transfection assay

JEG-3choriocarcinoma cells (American Type Cuture Collection) were cultured in minimal essential medium (MEM: Gibco-BRL) containing 10% fetal bovine serum. Twenty-four hrs prior to transfection, cells were plated onto 60-mm dishes. Two hrs before transfection, cells were cultured with fresh medium containing MEM, 10% charcoal stripped horse serum (SHS), and insulin (5 μg/ml). Transfection was carried out using 8 to 10 μg of DNA by the calcium phosphate method. After 24 hrs of transfection, the cells were washed twice with phosphate-buffered saline (PBS), and then the cells were treated with fresh medium (MEM plus 10% SHS and insulin), with or without ovine prolactin (1 μg/ml) and hydrocortisone (HC) (1 μg/ml). Twenty-four hrs after the treatment of hydrocortisone and prolactin, the cells were harvested, lysed with lysis buffer (Boehringer Mannheim), and assayed for luciferase and β-galactosidase activity.

Gel shift assays

Oligonucleotides encompassing the NFI palindromic site of the WAP CoRE (−820 to −720) and the adenovirus NFI consensus binding site were used for gel shift assays (coding strand for WAP NFI, 5′-TTGGGCACAGTGCCCAACAG-3′, and coding strand for adenovirus NFI, 5′-CTAGCTATTTTGGATTGAAGCCAATAT-3′. Equimolar concentrations of each oligonucleotide from both strands were annealed in the presence of 1X React 2 (Promega) buffer at 94° C for 10 min and then cooled to room temperature for 3-4 hr. The double-stranded oligonucleotide was end labeled with [γ-^{32}P] dATP using polynucleotide kinase (Gibco-BRL), and the probe was purified using p-6 Micro Bio-spin columns (Bio-Rad) followed by trichloroacetic acid precipitation to quantify the amount of labeled probe. The chimeric proteins (N-terminal of NFI-A4 fused with C-terminal of NFI-B2, N-terminal of NFI-B2 fused with C-terminal of NFI-A4) were expressed in JEG-3 cells, and nuclear extracts, isolated as described previously (Mukhopadhyay et al., 2001), were used for the DNA-binding assays. The amount of nuclear extract required for 50 % binding of the labeled probe was calculated, and then for each protein that amount was used for the competition assays. Nuclear extracts were isolated from the same passage of JEG-3 cells, and equimolar concentrations of each probe were used in the gel shift assays.

Antibodies

Both the chimera and wild type NFI constructs contained a hemoagglutinin (HA) epitope at their N termini. A monoclonal HA antibody (Babco, Berkeley, California) was used in the gel super shift assay to identify the HA-tagged NFI proteins. A monoclonal antibody raised against a synthetic peptide containing Serine-O-GlcNAc (Covance, Berkeley, California) detects Ser-O-GlcNAc and Thr-O-GlcNAc but did not show cross-reactivity with peptide determinants or

other closely related carbohydrate antigens. The monoclonal O-GlcNAc antibody was used in the gel super-shift assay to determine the serine and threonine-specific O-GlcNAcylation of the NFI proteins.

Co-immunoprecipitation

Cells were lysed with RIPA buffer and approximately 500 μg of lysate was incubated with 4-5 μl of antibody (HA and PRDX3) for 30 min on ice. 20 μl of protein A plus/protein G agarose beads (Oncogene) were incubated overnight at 4°C with constant shaking. Beads were washed with either PBS or TBST 3-4 times and then boiled with the sample buffer for 5 min followed by 10% SDS-polyacrylamide gel electrophoresis. Western blot was performed with HA antibody and then stipped [strip buffer (2%SDS, 50mM Tris pH 6.8, BME) at 55°C for one hr] the blot and re-probed with O-GlcNAc antibody.

Results

Determination of the transactivation and transrepression domain of the NFI A and B isoforms in the JEG-3 cell lines

In previous studies we determined that the specific NFI isoforms (A4, B2 and X1) were expressed at the mRNA level during lactation in the mouse mammary gland. Among the three isoforms, NFI-B showed the highest cooperativity with GR and STAT5A in the transcriptional activation of WAP CoRE assayed using transient transfection assays in JEG-3 cells. In contrast, the NFI-A4 isoform was inactive with GR and STAT5A on the WAP CoRE in JEG cells.

In order to determine the transacivation/repression domain of the NFI isoforms, we, therefore, generated chimeric proteins by making a fusion of the N-terminal region of the NFI-A isoform with the C-terminal region of the NFI-B isoforms and vise versa (N-AC-B and N-BC-A; Fig. 1B)). The chimeric proteins N-AC-B and N-BC-A (Fig.1B) were cloned into the mammalian expression vector pCH, and their activity on the pWAPtk-Luc reporter construct (Fig.1A) was determined. The prolactin receptor (PrlR), GR, STAT5A expression constructs were co-transfected along with the NFI-A, B and chimera constructs (N-AC-B and N-BC-A). pRSV-β-gal was used to monitor transfection efficiency and cells were induced with hydrocortisone (HC) and prolactin (Prl). The cooperativity of GR and STAT5A with NFI was only observed with NFI-B2 (7.5 fold, Fig1C), but not with NFI-A4 in confirmation of previous results [2]. However, interestingly the chimeric protein N-AC-B (N-ter of A4 and C-ter of B2, Fig.1C) resulted in a 9-fold induction of luciferase activity with GR and STAT5 as compare to the uninduced control. In contrast, HC and Prl did not increase luciferase activity of pWAPtkLuc in the presence of the chimeric construct N-B C-A (N-ter of B2 and C-ter of A4, Fig.1B),GR and STAT5A in JEG cells (Fig.1C). These experiments suggest that the C-terminal portion of NFI-B is the trans-activating region and that this region determines the cooperitivity between the GR, STAT5 and NFI protein in the JEG cells. On the other hand, the C-terminal portion of NFI-A4 did not display any cooperativity with GR, STAT5 and NFI, thus, the C-terminal region of NFI-A4 can be considered as a transrepression domain.

DNA-binding affinity of the chimera proteins to the palindromic site in the WAP CoRE

Previously we have shown that the DNA-binding specificity of NFI-B2 to the NFI palindromic site of the WAP CoRE is higher than the NFI-A4 by competition EMSA. We, therefore, compared the DNA-binding specificities between the chimeric transcription factors and their wild type counterparts by competition EMSA using oligonucleotides containing the WAP palindromic site and consensus NFI site of adenovirus replication origin region(Fig2). DNA-binding specificities of most of the NFI isoforms to the NFI site of the adenovirus replication origin have shown previously to be essentially equivalent [5]. Nuclear extracts were prepared from JEG-3 cells following transfection of NFI-A, B and their two chimeric constructs, as

described in Materials and Methods. The relative binding specificities were then estimated by measuring the amount of complex formation between the two NFI isoforms and the chimeric constructs and DNA as a function of probe DNA concentration using increasing amounts of the unlabeled probe as a competitor (Fig.2A and C). The results of these studies are summarized in Fig.2B and D. The DNA-binding specificities of the chimeric proteins N-BC-A and N-AC-B were equivalent to the wild type proteins NFI-B2 and NFI-A4, respectively. As the DNA-binding specificities of the chimeric proteins were not altered as compared to the wild type counterparts, this suggests that the C-terminal regions of the NFI proteins do not influence their DNA-binding specificity. These results also suggest that the DNA-binding specificity is not the "only" determining factor regulating the cooperative effects of the NFI-B2 isoform on the transcriptional activity of the WAP CoRE in JEG cells.

Serine and threonine-specific glycosylation (*O*-linked *N*- acetylglucosamine) on the C-terminal region of NFI-B isoform

The C-terminus is the most variable region of the NFI proteins, because it generated by alternative splicing. As cited previously, the C-termini of the NFI isoforms have already been demonstrated to act as a transactivating/suppressor domain. This region is highly proline-rich and also contains several serine and threonine residues as potential targets for either phosphorylation and/or *O*-glycosylation [5]. An involution-associated 74kDa NFI-C isoform has been identified which is post-translationaly modified by *N*-glycosylation [9,23,24]. We, therefore, compared the post-translation modification between the C-terminal regions of both the NFI-A4 and B2 to determine why the C-terminal region of NFI-B2, but not of A4 can activate the WAP CoRE in the JEG cells. We transfected the pCHNFI-A4, B2 and N-AC-B constructs into the JEG cells and EMSA supershift assays were performed using the cell extracts with oligonucleotides specific for NFI palindromic sequence of WAP and a serine/threonine-specific anti-O-GlcNAc monoclonal antibody (Fig.3). Since all the NFI constructs including the chimeric proteins were tagged with an HA epitope, we also employed an anti-HA monoclonal antibody in the EMSA assays (Fig.3, lane 1, 6 and 9) as a positive control. When we used the anti-*O*-GlcNAc monoclonal antibody in the supershift assay we observed the supershift of both proteins NFI-B2 (Fig.3, lane5) and the chimera N-AC-B (Fig3, lane8), but not the NFI-A4 protein (Fig3, lane3). This suggests that the C-terminus of NFI-B2 is probably post-translationally modified by *O*-glycosylation in the JEG cells.

This result was further confirmed by a co-immunoprecipitation assay (Fig4) with anti-HA and anti-*O*-GlcNAc antibodies. The constructs for the NFI-A, B and the chimeras NA-CB and NB-CA were transiently transfected to the JEG-3 cells. Proteins were immunoprecipitated first with the anti-HA antibody and blotted with the anti-HA antibody (Fig 4, upper panel). This blot then was reprobed with the anti-*O*-GlcNAc antibody and only the NFI-B (lane 2) and the chimera NA-CB proteins (lane 3) were detected. This result reconfirmed that the only C-terminal region of NFI-B is modified by *O*-glycosylation in the JEG cells.

Discussion

The NFI transcription factors have been shown by different groups including our own to be important factors required to maintain the regulated expression of several mammary gland-specific genes. Different splice forms of each NFI gene are expressed at the different stages of mammary gland development [5], and it is likely that each of these NFI isoforms may play an important role for the stage- and temporal-specific regulation of the mammary gland gene expression as well as in the regulation of mammary gland development. But the mechanisms by which these differential effects might be elicited are not well understood. Detailed analyses of the promoters and enhancers of the different mammary-specific genes suggest that

differential cooperativity with other transcription factors may determine the spatially- and temporally-restricted expression patterns of the mammary-specific genes.

The individual NFI proteins were initially thought to exhibit similar binding affinities to their cognate DNA binding site. However, we have shown previously that the NFI-B2 isoform displayed an increased binding affinity as compared to the NFI-X1 and NFI-A4 isoforms to the WAP promoter. Subsequent studies by other investigators have also revealed the differential binding affinity of specific NFI isoforms to their DNA binding motifs in other promoters [9]. While these differences in DNA binding affinity may help facilitate the specific competition of one NFI isoform with other NFI proteins, these differences alone may not be sufficient to account for their selective ability to cooperate with the other transcription factors for the regulation of the mammary-specific genes.

Posttranslational modifications of transcription factors play an increasingly important role in the regulation of gene transcription, especially to facilitate the interactions and cooperativity among multiple transcription factors and co-activators. Systemic hormones and growth factors in the mammary gland are likely candidates to regulate many of these post-translational modifications through downstream signal transduction pathways, and these modifications most likely will be critical regulators of mammary gland development. For example, the transcriptional activity of the NFI has been shown to be modulated by phosphorylation [25]. NFIC also has been reported previously to be modified by *N*- glycosylation [5]. Kane *et al.* have reported that a 74 kDa N-glycosylated NFIC isoform appears during mammary gland involution [9]. But the specific role of this N-glycosylated isoform is not well understood. These investigators have shown that this modification does not affect the ability of this NFIC isoform either to translocate to the nucleus or to recognize or bind to cognate NFI DNA-response elements. Our data also suggest that the DNA binding ability of the N-terminal region of both the chimeric proteins (Nter-A Cter-B and Nter-B Cter-A) was not altered by *O*-glycosylation (Fig.2). What then might be the function of *N*- or *O*-glycosylation of the NFI transcription factors? The *O*-glycosylation modification has been found in the C-terminal transactivation domain of the NFIB isoform suggesting that it might be influence the transcriptional activation domain. Most probably, these *N*- and *O*- glycosylation modifications may help the NFI isoforms, respectively, recruit the co-activators or co-repressors or to interact with other nuclear regulatory proteins. Recently STAT5 has shown to be modified by *O*-GlcNAc and this glycosylated form has been shown to bind with the CBP transcriptional coactivator. When the threonine 92 specific for the glycosylation of the STAT5 was mutated, this abolished transactivation of the target gene promoter.

In our previous study, we demonstrated that the NFI-B2 isoform exhibited the greatest cooperativity with STAT5A in transient transfection experiments. In contrast, the NFI-A4 isoform was ineffective in the transcriptional activation of the WAP distal CoRE. So, it is possible that the O-GlcNAc modifications of both the NFI-B2 and STAT5 are essential for the interaction between these two transcription factors either directly or indirectly, although this remains to be formally tested by site-directed mutagenesis. Notwithstanding, this is the first demonstration for WAP CoRE that the transcriptionally activate form of NFI not the repressor form is glycosylated,.

We previously proposed a working hypothesis that the accumulation of particular NFI isoforms coupled with the Prl-mediated activation of STAT5 and glucocorticoid activation of the glucocorticoid receptor at specific times during mammary gland development is critical for the appropriate temporal and spatial regulation of WAP gene expression. We now suggest that post-translational modification of both STAT5 and NFI-B2 isoforms by O-GlcNAc also might be critical to permit these transcription factors to activate WAP gene transcription during

mammary gland development. Further investigation will be required to identify the specific role of glycosylation of NFI-B in WAP gene transcriptional regulation.

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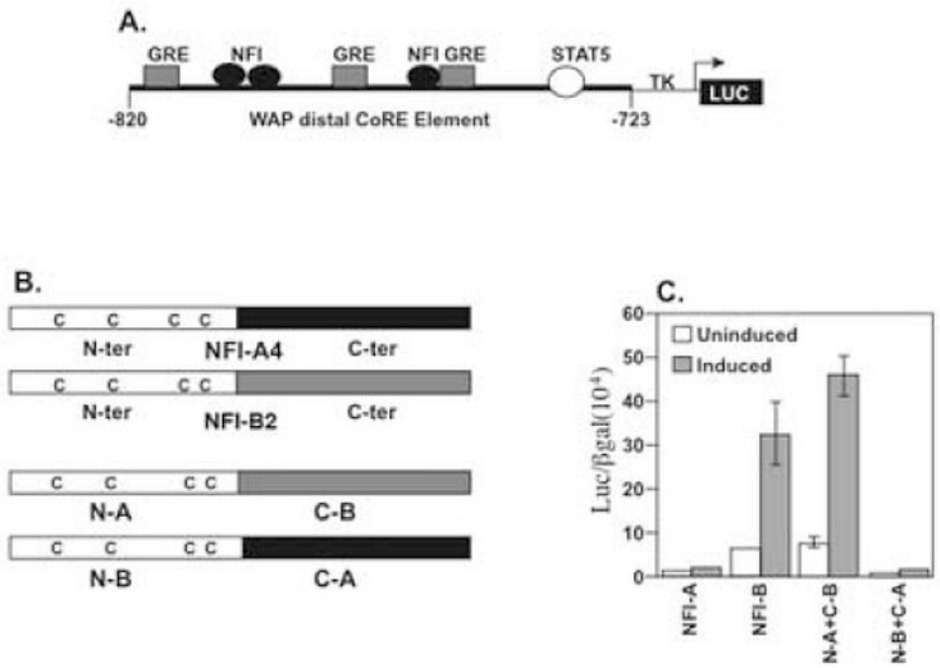

Figure 1.

The C-terminal portion of NFI-B2 activates the WAP CoRE. (A) Schematic diagram of the pWAPtk-luciferase construct. (B) Schematic diagram of the NFI-A4, NFI-B2 and the chimeric N-AC-B and N-BC-A. (C) The pWAPtl-luciferase construct (1μg) was transiently transfected into JEG-3 cells along with GR (0.2μg), STAT5A(1μg), PrlR (0.3μg), RSV-β-gal(0.3μg) and NFI isoforms (A4-0.2μg, B2-0.5μg) and both of their chimera constructs (NA-CB and NB-CA, 0.5μg of each). Twenty four hrs after cells were induced with both hormones HC and Prl (1μg/ml of each), luciferase expression was measured and transfection efficiencies were normalized to β-galactosidase activity. Error bars represent the standard error of three independent determinations.

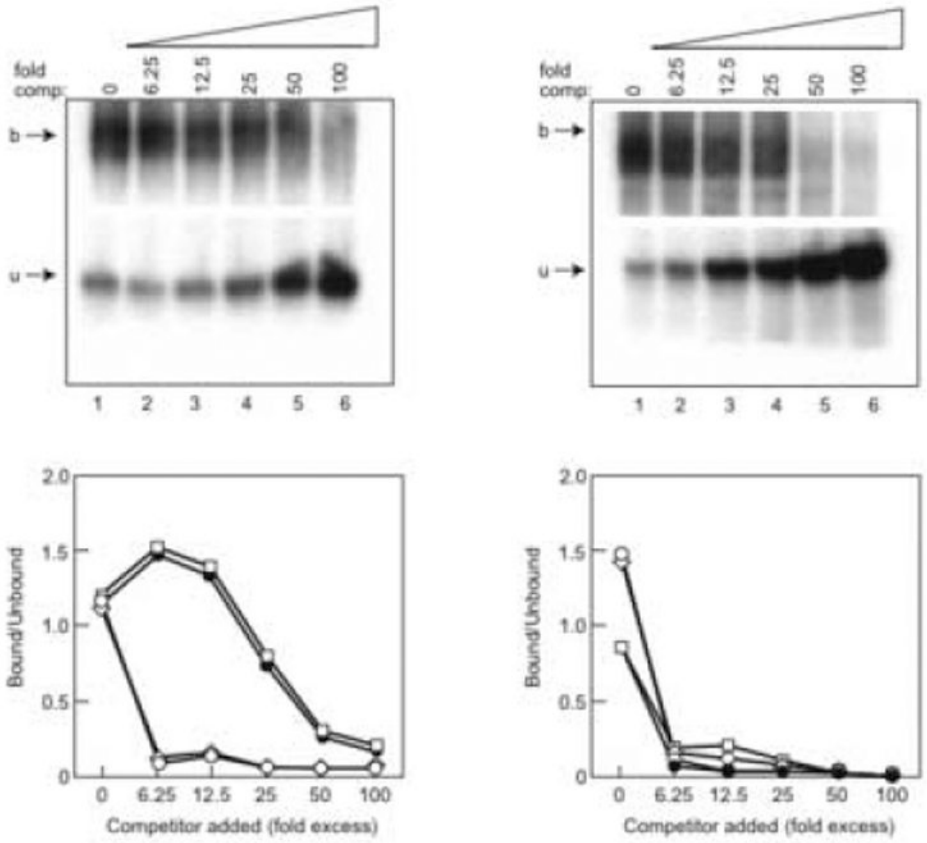

Figure 2.

DNA-binding specificities of NFI isoforms and their chimeric proteins. (A) DNA-binding specificity of NA-CB chimera to the palindromic motif present on the WAP distal promoter estimated by competition EMSA. The specific DNA-protein complexes (described in Materials and Methods) were made to compete with the same palindromic NFI oligonucleotide (homologous competitor) using increasing concentrations as shown (lanes 2 to 6; no competitor in lane1). (B) Comparison of DNA-binding specificity of the two NFI isoforms (A4 and B2) and their chimera proteins (NA-CB and NB-CA) to the WAP palindromic motif. The DNA-binding specificities of NB-CA, A4 and B2 were also determined along with the NA-CB (A) by competition EMSA, and the DNA-protein complex and free probe were quantitated by phosphoImager. Binding specificities were determined by plotting the percentage of bound/unbound versus competitor added. Open squares (□) represents the NFI-A4, black circle (●) represents the NA-CB, open circle (○) represents the NFI-B2 and open rectangle (◇) represents the NB-CA.(C) DNA-binding specificity of NA-CB of the chimera protein to the palindromic region of adenovirus origin of replication region estimated by competition EMSA. The specific DNA-protein complexes (described in the Materials and Methods) were made to compete with the same palindromic NFI oligonucleotide (homologous competitor) using increasing concentrations as shown (lane 2 to 6; no competitor in lane 1). (D) Comparison of DNA-binding specificity of the NFI-B2, A4 and their chimeric proteins to the palindromic region of the replication of origin of adenovirus. The DNA-binding specificities of NFI-B2, A4 and NB-CA were determined along with the NA-CB (C) by competition EMSA, and the DNA-protein complex and free probe were quantified by phosphoImager. Binding specificities were determined by plotting the percentage of bound/unbound versus competitor added. Open

squares (□) represents the NFI-A4, black circle (●) represents the NA-CB, open circle (○) represents the NFI-B2 and open rectangle (◇) represents the NB-CA.

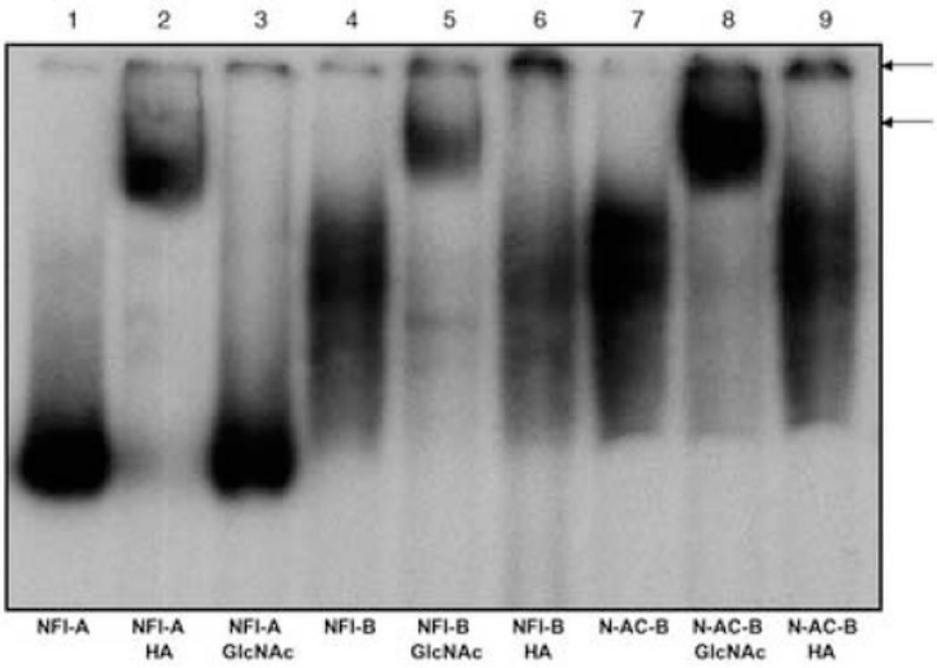

Figure 3.

Determination of glycosylation in the C-terminal domain of NFI-B2 isoform by EMSA supershift assay. NFI-B2, A4 and the chimeric NA-CB were transiently transfected into JEG-3 cells and the proteins were incubated with the labelled palindromic motif present on the WAP distal promoter to form a DNA-protein complex (described in the Material and Methods; lanes 1-NFI-A, lane 4-NFI-B and lane 7-NA-CB). 1 μg of each of anti-HA (lanes 2, 6 and 9) and anti-Serine-O-GlcNAc (lanes 3,5 and 8) antibodies were added in the DNA-protein complex. Both the arrows represent the supershift complexes.

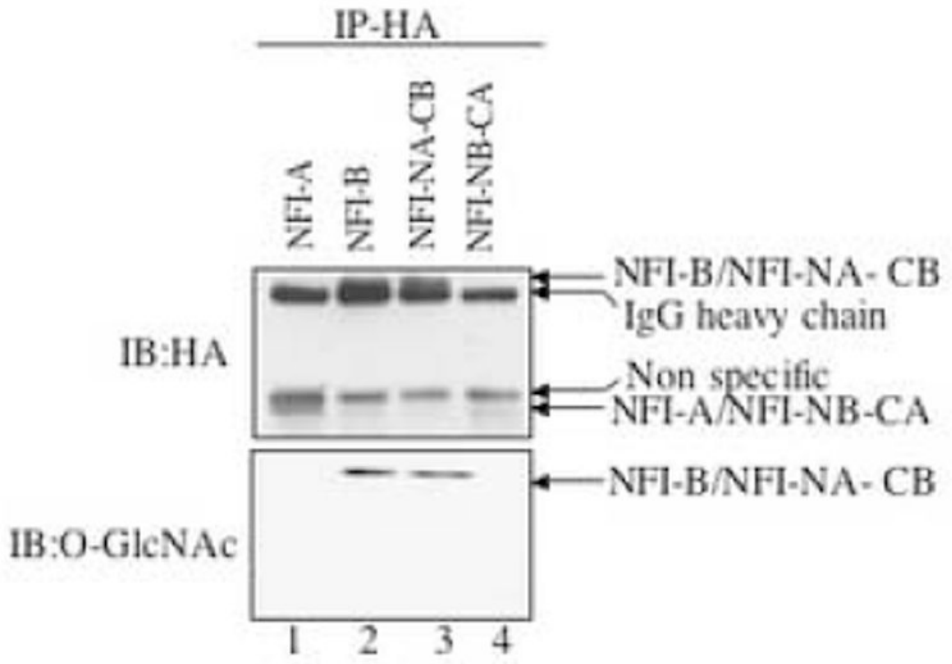

Figure 4.

Confirmation of glycosylation on the C-terminal region of the NFI-B isoform by co-immunoprecipitation. NFI-A, B and both of the chimeric constructs (NA-CB, NB-CA) were transiently transfected into the JEG-3 cells and the total proteins were precipitated with the HA antibody and blotted with an anti-HA antibody (upper panel). The blot was reprobed with the anti-Serine-O-GlcNAc antibody (lower panel).