Nuclear factor-I and activator protein-2 bind in a mutually exclusive way to overlapping promoter sequences and *trans*-activate the human growth hormone gene

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ABSTRACT

Transcription of the human growth hormone (hGH) gene and its regulation are controlled by *trans*-acting factors that bind to hGH gene promoter sequences. Several DNase I footprints have been described within 500 bp of this promoter, one of which (–289 to –267) has not yet been ascribed to a defined factor. By DNase I footprinting, gel mobility shift, and methylation interference assays with extracts from HeLa cells and GH-producing pituitary tumor (GC) cells, we show that this factor belongs to the NF-I family. When NF-I was competed out of the cell extracts, the *trans*-acting factor AP-2 bound to the same site as NF-I. AP-2 was present not only in HeLa cells, but also in GC cells albeit at a much lower concentration. Consistent with the mutually exclusive binding of NF-I and AP-2, their methylation interference patterns included four guanine residues that were crucial for binding of both NF-I and AP-2. Cell-free transcription from the hGH gene promoter showed that these two factors can *trans*-activate this gene.

INTRODUCTION

The growth hormone (GH) gene is an interesting model for studying the control of gene expression by hormones and *trans*-acting factors. The human hGH-N (hGH-1) gene is expressed exclusively in the somatotrophs of the anterior pituitary. Its transcription is stimulated by GHRH via cAMP and by glucocorticoids (1). The mechanism of these hormonal effects, which probably involve *cis*-acting hGH gene promoter sequences, is ill-understood. The tissue-specificity of expression of the hGH gene is ascribed, at least in part, to a pituitary-specific factor called GHF-1/Pit-1 (2). This homeobox-containing protein (3) binds to two contiguous sites on the promoter, thereby protecting these sites against cleavage by deoxyribonuclease I (DNase I) (footprints –92 to –65 and –130 to –105).

Other factors, which also occur in nonpituitary cells such as HeLa cells, bind to the hGH gene promoter and may contribute to controlling its basal and (or) hormone-dependent transcription.

The interaction of some of these factors is revealed by the DNase I footprints observed on the hGH gene promoter when it is incubated with extracts from pituitary tumor (GC) cells or HeLa cells. One of these footprints, called growth hormone footprint 2 (GHF2), extends from –139 to –115 and is due to the binding of a factor indistinguishable from Sp1 (4). Another footprint called GHF3 extends from –289 to –253. This footprint is due to factor(s) that appears to contribute to the control of hGH gene transcription (5). While the proximal part of GHF3 (GHF3p, –266 to –253) reflects the binding of upstream stimulating factor/major late transcription factor (USF/MLTF) (4), the distal part of this footprint (GHF3d) has not yet been ascribed to a particular factor. Although activator protein 2 (AP-2) purified from HeLa cells had been reported (6) to bind on the hGH gene promoter from –287 to –265, we had shown (4) that GHF3d is probably due to another factor. We have now investigated this question by DNase I footprinting, gel mobility shift, and methylation interference assays. We have also evaluated the functional significance of factor(s) binding to GHF3d by cell-free transcription experiments.

MATERIALS AND METHODS

Materials

Enzymes were purchased from Promega Biotec or Boehringer. Radiolabeled compounds and reverse transcriptase (AMV) were from Amersham International plc. Poly(dI-dC) was from Sigma, dimethylsulfate from Aldrich, and piperidine from Merck. Tissue culture components were from Flow Laboratories or from Gibco BRL.

Oligonucleotides

The following double-stranded oligodeoxyribonucleotides (oligo) were from Eurogentec (Liège, Belgium) except oligo AP-2 which was a gift from Dr. D. Christophe (Free University of Brussels). Oligo NF-I corresponds to the high-affinity binding site (+21 to +44) in the adenovirus origin of replication, modified in such a way (underlined) that a putative AP-2 consensus sequence is eliminated, 5' ATTTTGGCTACAAGCCAATATGAT 3'. Oligo

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GHF3d corresponds to the distal moiety (−289 to −267) of GHF3, 5' ACCATGGCCTGCGGCCAGAGGGC 3', plus a 5' TCGA end. Oligo AP-2 corresponds to the high-affinity AP-2 binding site on the human metallothionein-IIA gene from −188 to −166, 5' GAACTGACCGCCCGCGGCCCGTG 3', plus a 5' AT end and a 3' CATG end on the coding strand only. Oligo GHF-1 corresponds to the proximal GHF-1/Pit-1 binding site on the hGH gene promoter (−88 to −69) plus *Bam*HI compatible ends, 5' GATCCCATGCATAAATGTACACAG 3'.

Plasmid constructions

Plasmid pHGH494 Δ−260/−188 Δ−149/−57 contains a 497-bp *Eco*RI-*Bam*HI (−494 to +3) insert of the hGH-1 gene in pBR322 with deletion of nucleotides −260 to −188 and −149 to −57. This construction is convenient to visualize the footprints described. Plasmid pHGH37-L, used as an internal control for cell-free transcription, contains a 40 bp *Hae*III-*Bam*HI (−37 to +3) insert of the hGH-1 gene in pBR322 with insertion of a *Bam*HI-*Sma*I linker downstream from +3. The deletion mutant p13-13 (7) contains 237 bp of the long terminal repeat (LTR) of the mouse mammary tumor virus (MMTV) upstream of the transcription initiation (cap) site.

Cells and cell extracts

Human cervix carcinoma (HeLa S3) and rat pituitary tumor (GC) cells were grown in modified Eagle's medium for suspension cultures, supplemented with 10% newborn calf serum (HeLa cells) or with 5% foetal calf serum plus 12.5% horse serum (GC cells). Whole cell extracts (10–20 mg protein/ml) were prepared as described (8), except that 0.75 instead of one volume of saturated ammonium sulfate solution was added for the first precipitation step of GC cell extracts.

Binding assays

DNase I footprinting and gel mobility shift assays were performed as described (4). For the methylation interference assay, the procedure of Chodosh *et al.* (9) was used with minor modifications. Single stranded oligodeoxyribonucleotides were labeled with γ -[³²P]-ATP in the presence of T₄ polynucleotide kinase and hybridized with the unlabeled complementary strand as described (4). Probes were partially methylated with dimethylsulfate and used in a binding reaction, with the modification that each incubation was scaled up to 30 μ l and contained 50,000 cpm of the appropriate probe, 8 μ g of poly(dI-dC) and 120 μ g of cell extract protein. For NF-I contact points analysis, all incubations also contained 50 ng (GC extracts) or 150 ng (HeLa extracts) of oligo AP-2 to prevent binding of this factor to the radioactive probe. For AP-2 contact points analysis, the incubation contained 100 ng of oligo NF-I to prevent binding of NF-I to the radioactive probe. After incubation at room temperature for 30 min, samples were loaded on a low ionic strength 5% polyacrylamide gel and electrophoresed according to Carthew *et al.* (10). Overnight autoradiography at 4° C identified free DNA (F) and bound protein-DNA complexes (B) which were isolated from the gel matrix by horizontal migration onto NA-45 DEAE membranes (Schleicher and Schuell). DNA was eluted as described (11), phenol/chloroform extracted, and purified by anion exchange chromatography (Qiagen-tip 5). After two ethanol precipitations, the DNA was piperidine-cleaved, lyophilized, electrophoresed on a 18% polyacrylamide-8 M urea gel, and visualized by autoradiography.

Cell-free transcription and primer extension

For *in vitro* transcription experiments, each incubation contained 100 μ g of cell extract protein, 200 ng of circular template pHGH494 Δ−260/−188 Δ−149/−57 and 150–200 ng of circular pHGH37-L template used as internal control, in 10 μ l of 20 mM Tris.Cl (pH 7.9), 50 mM KCl, 6.25 mM MgCl₂, 0.05 mM EDTA, 1 mM dithiothreitol and 8.5% glycerol. Incubations lasted for 90 min at 30 °C. After DNase I treatment RNA's were hybridized with 30,000 cpm of labeled primer in 15 μ l of 50 mM Tris.Cl (pH 8.0), 50 mM KCl, 8 mM MgCl₂, 4 mM dithiothreitol and were heated for 2 h at 60° C. The primer, complementary to nucleotides 436 to 418 of pBR322, was labeled with γ -[³²P]-ATP in presence of T₄ polynucleotide kinase. The duplexes were incubated at 42° C for 30 min in the same buffer plus 1 mM dNTP, 80 μ g/ml of actinomycin C1 and 11.5 units of AMV reverse transcriptase. Nucleic acids were analyzed on an 8 M urea, 8% polyacrylamide gel, autoradiographed and quantified by densitometry (Joyce Loeb Chromoscan 6). Transcripts from the two templates were distinguishable because those from pHGH37-L were 12 bp longer, due to the insertion in the latter of the *Bam*HI-*Sma*I linker.

RESULTS

NF-I interacts with the hGH gene promoter

Examination of the sequence corresponding to GHF3d (Table 1) shows that it contains two overlapping putative binding sites, one for AP-2 and one for nuclear factor I (NF-I). Each of these sequences contains one mismatch with the consensus. The AP-2 mismatch does not prevent binding of purified AP-2 (6). We therefore investigated whether NF-I can also bind to this region of the hGH gene promoter. NF-I actually refers to a family of ubiquitous nuclear proteins which are differently expressed depending on the tissue and species (12). The footprints shown in Fig. 1 demonstrate that our cell extracts contained NF-I and that the latter can bind to the GHF3d sequence. The promoter of the LTR of MMTV known to contain a high affinity binding site for NF-I (13) was incubated with HeLa (lanes 2–5) or GC (lanes 6–9) cell extracts. A typical NF-I footprint was observed (lanes 2 and 6) at the expected coordinates, *i.e.* from −81 to −58 relative to the cap site. This NF-I binding was prevented by competing oligo GHF3d (lanes 3 and 7), but not by addition of two unrelated oligos such as AP-2 (lanes 4 and 8) or USF (lanes 5 and 9). We conclude that NF-I can recognize the GHF3d sequence despite the mismatch with the consensus.

Gel mobility shift assays provided additional evidence for the binding of NF-I to the hGH gene promoter from −289 to −267. When labeled oligo GHF3d was incubated with HeLa or GC cell extracts a wide retarded band was observed, corresponding to protein-GHF3d interactions (Fig. 2, lanes 2 and 5). A 50-fold molar excess of unlabeled oligo GHF3d inhibited the formation of this complex (lanes 3 and 6), which was therefore considered as specific. Indeed, an unrelated oligonucleotide (oligo USF) was unable to prevent the formation of this oligo GHF3d-protein complex (lanes 4 and 7). The width of this specific complex could reflect the polypeptidic heterogeneity of NF-I which generates closely migrating bands in gel mobility shift experiments (14). The fact that this interaction persisted under high salt conditions (150 mM KCl) is also in favor of complexes involving NF-I (14, 15). A qualitative difference was seen between the data obtained with HeLa and GC cell extracts. The specific complex produced in HeLa cell extracts migrated more slowly than the one produced

Table 1. Comparison of the hGH gene promoter sequence (GHF3d) protected against DNase I cleavage by GC and HeLa cell extracts, with the consensus binding sites for AP-2 and NF-I.

AP-2 consensus	5'	GCCTGGGG	3'
GHF3d	5' (-289)	ACCATGGCCTGCGGCCAGAGGGC	(-267) 3'
		c	
NF-I consensus	5'	TGG NNNNNGCCAA	3'
		A	

Identities between sequences are indicated by vertical bars. N refers to any nucleotide. The consensus for AP-2 and NF-I are taken from refs. 20 and 15, respectively.

in GC cell extracts (compare lanes 2 and 5 in Fig. 2). This is consistent with the different tissue and species expression of the polypeptides of the NF-I family, but does not rule out the involvement of unrelated proteins, since different factors can bind to a same DNA sequence (16). We investigated this question by methylation interference experiments.

Despite their heterogeneity, the members of the NF-I family share the same DNA binding domain (17, 18) and are therefore expected to produce the same methylation interference pattern on their specific DNA target. Double-stranded oligo GHF3d labeled at one 5' end was partially methylated with dimethylsulfate and used in a binding reaction with HeLa or GC cell extracts. Despite the difference seen in band shift between HeLa and GC cell extracts, the methylation interference pattern was identical with these two extracts (Fig. 3). Data with the coding (Fig. 3A) and the noncoding (Fig. 3B) strands show that the guanines that are essential for the interaction are located exclusively in the two boxes of the palindromic NF-I consensus sequence. On the coding strand and in the 5' half palindrome of the noncoding strand, these guanines correspond (Table 2) to those described to be crucial for the interaction between NF-I and its binding site on the adenovirus replication origin (19). We conclude that polypeptides of the NF-I family present in HeLa and GC cell extracts interact with the same nucleotides of the sequence from -289 to -267 of the hGH gene promoter.

Mutually exclusive binding of NF-I and AP-2

To investigate the possible involvement of AP-2 in the GHF3d seen with GC cell extracts, we first determined whether this protein was present in such extracts. Indeed, AP-2 is not strictly tissue-specific (20) and has not been described in pituitary cells. Gel mobility shift experiments were performed with labeled oligo GHF3d incubated with GC cell extracts in the presence of competing oligo NF-I to prevent binding of NF-I to GHF3d (Fig. 4A). Three retarded bands were seen (lane 5), one of which (arrow) disappeared when the incubation was conducted in presence of oligo AP-2 (lane 6). This band was therefore considered as revealing a specific interaction between AP-2 and the GHF3d sequence. The retarded bands which did not disappear under competition conditions were considered as nonspecific. The very weak intensity of the AP-2 specific band as compared to that with HeLa cell extracts (see Fig. 4B below) probably reflects

Fig. 1 DNase I footprinting of NF-I on the LTR of MMTV. The DNA probe consists of a 438 bp-long *Bam*HI-*Rsa*I fragment of p13-13 labeled at the *Bam*HI end of the coding strand. The probe was incubated without (lane 1) or with 45 μ g HeLa (lanes 2-5) or 60 μ g GC (lanes 6-9) cell extract protein. A 200-fold excess (30 ng) of unlabeled oligo GHF3d or oligo AP-2 or (unrelated) oligo USF was present in the incubations as indicated above the lanes. The radioactive size marker lane (M) is pBR322 digested by *Hpa*II.

a low relative concentration of AP-2 in GC cell extracts. The AP-2 complex observed with GC and HeLa extracts migrated with the same mobility.

The coexistence of AP-2 and NF-I in our cell extracts raised the question of their simultaneous or mutually exclusive binding on the target DNA sequence, the more so as most of the nucleotides involved in the AP-2 consensus lie between those of the NF-I consensus (Table 1). To approach this question, we first performed gel mobility shift assays with labeled oligo GHF3d

incubated with GC (Fig. 4A) or HeLa (Fig. 4B) cell extracts in the absence or presence of competing unlabeled oligonucleotides. With HeLa cell extracts, a specific oligo GHF3d-protein complex was seen (Fig. 4B, lane 2), which disappeared with competing oligo GHF3d (lane 3), but not with an unrelated (USF) oligo (lane 7), as expected. Addition of competing oligo AP-2 did not modify this specific complex (lane 4). In contrast, incubation with competing oligo NF-I led to the disappearance of the GHF3d-protein complex and the appearance of a narrower band in the same region of the gel (lane 5). This new complex was AP-2 specific since it disappeared when oligo AP-2 was added to the

incubation containing oligo NF-I (lane 6). The same pattern of specific complexes was obtained with these competing oligonucleotides in GC cell extracts (Fig. 4A). These experiments suggest that the specific GHF3d-protein complex seen in our gel mobility shift assays results from an interaction with NF-I. The binding of AP-2 on the GHF3d sequence can be detected only when binding of NF-I is prevented. Thus, the binding of NF-I and AP-2 appears to be mutually exclusive in our experimental conditions.

If the GHF3d footprint does correspond to the binding of either NF-I or AP-2, then the methylation interference pattern of the binding observed in absence of NF-I should involve the AP-2 consensus. The methylation pattern observed when NF-I was titrated out of the incubation (Fig. 5 and Table 2) was indeed clearly different from that detected by NF-I contact point analysis. Six of the seven guanines that were important for the interaction of the protein with the two DNA strands are part of the AP-2 consensus. The seventh guanine (noncoding strand) is just next to the 5' end of this consensus.

Gel mobility shifts with the isolated oligo GHF3d sequence do not necessarily reflect the situation with the intact promoter. Indeed, DNA-protein interactions may be influenced by flanking promoter sequences. To evaluate the actual contribution of NF-I and AP-2 to the GHF3d, we performed the DNase I footprinting experiments shown in Figure 6. The expected GHF3 footprint was seen on the hGH gene promoter with the two cell extracts (lanes 2 and 6). To visualize only GHF3d, competing oligo USF was included (lanes 3–5, 7–9). When competing oligo AP-2 was added (lanes 3,7) the GHF3d footprint was not modified, as described earlier (4). This ruled out the direct involvement of AP-2 in GHF3d under these conditions. With HeLa cell extracts, addition of a 200-fold excess of competing oligo NF-I instead of oligo AP-2 (lane 4) did not completely prevent the footprint either, as if another protein were now interacting with the promoter. This protein was AP-2 since concomitant addition of competing oligo NF-I and oligo AP-2 totally prevented this

Fig. 2 Detection of protein-oligo GHF3d complexes by gel mobility shift assay. Labeled oligo GHF3d was incubated without (lane 1) or with 20 μ g HeLa (lanes 2–4) or 20 μ g GC (lanes 5–7) cell extract protein. A 50-fold excess (15 ng) of unlabeled oligo GHF3d or (unrelated) oligo (USF) was added as indicated above each lane. The specific protein-DNA complexes are indicated in the margin. All incubations contained 150 mM KCl.

Fig. 3 Methylation interference analysis of NF-I/DNA interactions at the GHF3d site. Oligo GHF3d labeled either on the coding strand (A) or on the noncoding strand (B) was incubated with HeLa or GC cell extracts as described in Materials and Methods. Coding and noncoding sequences are shown along the gel. The consensus sequence for NF-I is boxed, with the mismatch indicated by the dashed line. Methylated residues that interfere with the binding to the recognition sequence are indicated by arrowheads. F refers to free DNA and B refers to bound DNA.

Table 2. Methylation interference patterns for NF-I and AP-2 binding.

Adeno- virus	(+20)	▽▽ ▽	TATTTTGGATTGAAGCCAATATGATAATGA (+49) ATAAAACCTAACTTCGGTTATACTATTACT	(+49)
		△△ △△		
hGH	(-289)	▽▽ ▽	ACCATGGCCTGCGGCCAGAGGGC (-267) TGGTACCGGACGCCGGTCTCCCG	(-267)
		○ ○○ △ △△		

Triangles refer to NF-I and circles to AP-2. The filled circle corresponds to partial interference. Data for the adenovirus are taken from de Vries *et al.* (19). The NF-I consensus sequence is underlined, and the AP-2 consensus is in bold characters.

Fig. 4 Mutually exclusive binding of NF-I and AP-2 to their target sequence demonstrated by gel mobility shift assay. Labeled oligo GHF3d was incubated without (lane 1, A and B) or with 20 μ g GC (A, lanes 2 to 6) or 20 μ g HeLa (B, lanes 2 to 7) cell extract protein. 15 ng of unlabeled competing oligonucleotide were added as indicated. The arrow in the margin (A) points to the specific complex between AP-2 and DNA (lane 5).

footprint (lane 5). With GC cell extracts, the mere addition of oligo NF-I sufficed to prevent GHF3d (lane 8). Because of its much lower abundance in these extracts than in HeLa cell extracts, AP-2 was unable to produce a footprint even when NF-I was titrated out (compare lane 8 with lane 4). Therefore, the same DNase I pattern was seen with a combination of oligo AP-2 and oligo NF-I (lane 9) as with oligo NF-I alone (lane 8). The same patterns of GHF3d in such competition experiments were observed also in absence of oligo USF (not shown), suggesting that the interaction of NF-I and AP-2 with their respective binding sites does not depend on binding of USF to the neighboring sequence -266 to -253. These results confirm the data obtained

by gel mobility shift assay, namely that AP-2 and NF-I can bind independently to the hGH gene promoter. In our experimental conditions, when AP-2 and NF-I coexist, NF-I interacts preferentially with this promoter.

Functional studies

Cell-free transcription experiments were performed to determine how binding of NF-I or AP-2 affected the activity of the hGH gene promoter (Fig. 7). The template used contained the pHGH494 Δ -260/-188 Δ -149/-57 promoter. The deletion Δ -149/-57 eliminates the binding site for the pituitary-specific *trans*-acting factor GHF-1/Pit-1 and for the ubiquitous factor Sp1,

Fig. 5 Methylation interference analysis of AP-2/DNA interactions at the GHF3d site. The consensus sequence for AP-2 is boxed. The open arrowhead corresponds to partial interference. See Fig. 3 for other details.

Fig. 6 Mutually exclusive binding of NF-I and AP-2 on the hGH gene promoter demonstrated by DNase I footprinting. The DNA probe consists of a 330 bp-long *EcoRI-BamHI* fragment of phGH494 Δ -260/-188 Δ -149/-57 labeled at the *BamHI* end of the coding strand. The probe was incubated without (lane 1) or with 40 μ g HeLa (lanes 2-5) or 55 μ g GC (lanes 6-9) cell extract protein. A 200-fold excess (30 ng) of unlabeled oligonucleotides was added in the incubations as indicated above the lanes.

so that effects of other factors can be more conveniently analyzed. The deletion Δ -260/-188 affects none of the footprints seen on the hGH gene promoter when incubated with GC or HeLa cell extracts. When transcribed in a GC cell extract, the construct

Fig. 7 Cell-free transcription of phGH494 Δ -260/-188 Δ -149/-57 *i.e.* devoid of GHF-1/Pit-1 sites (test template) in GC cell extracts. All incubations contained phGH37-L (control template) and 100 ng of oligo NF-1 (lane b) or oligo AP-2 (lane c) or both (lane d). Control incubations (lane a) contained 100 ng of oligo GHF-1. DNA concentration in all incubations was adjusted to the same level by adding poly(dI-dC). The relative transcriptional activity was calculated by densitometry. The data were normalized for transcription from the control template and expressed relative to transcription in control incubations (lane a). Data are means $\pm$ S.E.M. for three independent experiments.

generated transcripts initiated from around the cap site determined *in vivo* (21), namely at nucleotides +1 and +2. These transcripts were RNA polymerase II-dependent since they disappeared in presence of α -amanitin (not shown). When the experiment was conducted in presence of competing oligo NF-I, there was a 40% decrease in transcriptional activity. Because the template DNA/protein ratio is much higher in cell-free transcription than in binding assays, AP-2 was expected to interact with the templates even in the presence of NF-I. Indeed, addition of competing oligo AP-2 led to a decrease of 30% in transcriptional activity. One would therefore predict that transcriptional activity is further decreased in presence of a mixture of competing oligo NF-I and AP-2. Data in Fig. 7 show that this was the case since transcription was now reduced by about 50%. We conclude that NF-I and AP-2 both can *trans*-activate the hGH gene promoter. The residual transcriptional activity observed under these conditions may be ascribed to untitrated factors, including USF/MLTF which is known to bind to this construct (4).

DISCUSSION

We have studied here the cell-free binding of transcription factors to the hGH gene promoter sequence from -289 to -267 (GHF3d), the only footprint within 0.5 kb of this promoter which has not yet been ascribed to a defined factor. By DNase I footprinting and gel mobility shift assay, we have shown that this sequence can bind Nuclear Factor I (NF-I)/CCAAT Transcription Factor (CTF) in a specific way. NF-I and CTF have been independently described but actually refer to the same family of factors (14). NF-I was originally purified from HeLa cells as a DNA-binding protein required for efficient adenovirus replication *in vitro* and *in vivo* (14, 15). CTF was known for its ability to activate several eukaryotic promoters (14). Using methylation interference, de Vries *et al.* (19) have identified on the adenovirus origin of replication the NF-I contact points with

the DNA bases shown in Table 2. This raised the question of the actual contact points of NF-I with an eukaryotic promoter such as that of the hGH gene. The five guanines that are involved in the interaction of NF-I with the adenovirus sequence (19) were found here as being important also in the hGH gene sequence (Table 2). Methylation of a guanine on the hGH gene noncoding strand (−282) prevented NF-I binding. This guanine corresponds to a thymine (+28) on the noncoding strand of the adenovirus sequence. Thus, replacement of this adenovirus pyrimidine by a purine does not prevent NF-I binding, and this confirms BrdU-substitution data (19). In contrast, methylation of the guanine at position −281 on the noncoding strand did not interfere with NF-I binding to the hGH gene sequence, despite the fact that methylation of the corresponding adenine at position +29 of the adenovirus did (Table 2). This is consistent with the fact that dimethylsulfate methylates guanines on the N7 atom which is in the major groove and adenines on the N3 atom which is in the minor groove of the DNA helix. This confirms that NF-I faces the minor groove at coordinate +29 of the adenovirus and suggests that it is also the case at coordinate −281 of the hGH gene promoter. The guanines at −272 and −277 on the coding strand of the hGH gene sequence replace the adenines at +38 and +33 of the adenovirus sequence. Methylation of these guanines did not interfere with NF-I binding. The electrophoretic mobilities of the complexes between NF-I and its target sequence differed depending on the origin of this factor *i.e.* HeLa or GC cells (Fig. 2), and yet the methylation pattern was identical (Fig. 3). This is compatible with the finding (12, 17, 18) that different tissues may express different isoforms of NF-I all sharing the same DNA binding domain. We conclude from these experiments that the molecular mechanisms of DNA recognition by NF-I are similar when this factor binds to the origin of replication of adenovirus and to the hGH gene promoter. This is consistent with the recent finding that the same domain of NF-I mediates DNA binding on the adenovirus origin of replication and on an eukaryotic promoter (22).

AP-2 purified from HeLa cells protects two regions of the hGH gene promoter against cleavage by DNase I (6). The first region (−167 to −145) binds AP-2 with low affinity and the other (−287 to −265), which corresponds approximately to GHF3d (−289 to −267), binds AP-2 with much higher affinity. However, with crude HeLa cell extracts, no footprint was seen at the low affinity site (5) and we show here with such extracts that the footprint located at the high affinity site is in fact due to NF-I. When the latter was titrated out of these cell extracts, AP-2 did produce an identical footprint, consistent with the mutually exclusive binding of AP-2 and NF-I to this site. By gel mobility shift assay, we have demonstrated that AP-2 is also present in GC cell extracts, but at a much lower concentration than in HeLa cell extracts. Indeed, no AP-2 footprint was seen from −289 to −267 when NF-I was titrated out of GC cell extracts. Methylation interference experiments showed that six guanines that were important for the interaction of AP-2 with the two DNA strands are part of the AP-2 consensus. Our identification of a crucial guanine residue just next to the AP-2 consensus is not surprising since the length of this consensus varies from 8 to 10 bp (20, 6). Our data also show that methylation of four guanine residues, namely at −276 and −283 on the coding strand and −275 and −282 on the noncoding strand, prevented binding of both NF-I and AP-2. This supports the mutually exclusive character of the binding of these factors on the hGH gene promoter. There are other cases where AP-2 or a CCAAT-transcription factor is displaced by another protein.

A CCAAT-binding transcription factor that interacts with a sea urchin histone 2B-1 gene promoter is competed for by a sperm-specific CCAAT displacement protein which might act as a repressor of gene transcription (23). Another example is the displacement of AP-2 by AP-3 from its binding site on the SV40 core sequence (24).

As to the functional significance of NF-I binding, our cell-free transcription data suggest that this factor stimulates transcription from the hGH gene promoter. This interpretation is in agreement with transfection data from Lefevre *et al.* (5). One of their hGH gene promoter mutants called $\Delta Nco2$ -CAT that lacked a region encompassing the GHF3d sequence was less active in terms of CAT activity than the intact promoter. Moreover, two other hGH gene promoter mutants (hGH-NK and hGH $\Delta Nco1$) that had an altered GHF3d sequence, and yet still showed a footprint, were as active as the intact promoter in the CAT assay. This behavior can now be explained in light of our results since these mutants still contain a NF-I consensus. NF-I is also known to be involved in the glucocorticoid-dependent stimulation of transcription from the MMTV promoter (25,26). Schüle *et al.* (27) and Strähle *et al.* (28) showed that NF-I can interact synergistically with the glucocorticoid receptor to stimulate MMTV transcription. Since this receptor also binds to the hGH gene (29,30), NF-I could play a similar role in controlling glucocorticoid-dependent transcription of this gene. Concerning AP-2, this relatively cell-specific factor is involved in the basal control of promoters such as that of the metallothionein-IIA gene where it mediates transcriptional activation by phorbol esters and cAMP (6). A role of AP-2 in the cAMP-dependent transcriptional activation of the hGH gene by GHRH was therefore an interesting possibility. However, this hypothesis has been recently discounted by experiments with hGH gene promoter deletion constructs (31). Still, our cell-free transcription experiments suggest that AP-2 can *trans*-activate the hGH gene, which is therefore a candidate for regulation by this factor *in vivo*. Transcription of the rat GH gene is stimulated by retinoic acid through 5' flanking *cis*-acting sequences (32). Since retinoic acid increases the concentration of AP-2 mRNA (33), a role of AP-2 in this control mechanism cannot be excluded.

The cloning of NF-I and AP-2 has shown that their DNA-binding domains share no structural features with each other or with other *trans*-acting factors (22). However, NF-I and AP-2 have in common a proline-rich domain which appears to be indispensable for their transcriptional activity (22). Since NF-I and AP-2 can *trans*-activate the hGH gene by binding to the same promoter region, the interaction of this proline-rich domain with a specific component of the transcriptional machinery might involve a *trans*-activating mechanism that is common to NF-I and AP-2.

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