Expression of hst (k-FGF, FGF-4), a member of the fibroblast growth factor gene family, is restricted to early stages of developing embryos and to embryonal carcinoma cells. In F9, which is a prototype of embryonal carcinoma cells expressing hst, the expression of hst gene is positively regulated by a downstream octamer motif that functions as an enhancer. We have investigated, by chloramphenicol acetyltransferase (CAT) reporter fusion gene analysis in F9, the cis-acting regulatory element within the hst promoter region that interacts with this enhancer. Electrophoretic mobility shift assay and methylation interference analysis showed that the hst promoter contains, in a segment termed Y, the sequence 5'-CTGATTGGCAGGCGG-3', which closely resembles the consensus binding motif for the CCAAT-binding factor NF-Y. Deletions or mutations in this element substantially reduced expression of hst-CAT constructs. The nuclear factor binding to the Y segment of the hst promoter was indistinguishable from NF-Y, as inferred from interactions with specific anti-NF-Y monoclonal and polyclonal antibodies. We conclude that the expression of the hst gene in F9 is positively regulated by the coordinated interaction between an NF-Y-binding site and an octamer motif.

Transcriptional regulation is one of the crucial steps determining which gene should be selectively switched on or off. Considerable evidence implies that this is dictated by trans-acting factors that interact with cis-elements acting within transcription units and that it is the combined action of such sequence-specific DNA-binding proteins that confers on a given gene its particular pattern of transcriptional activity (1).

F9 is an embryonal carcinoma cell line resembling the inner cell masses of blastocysts both morphologically and antigenically (2). Differentiation of F9 can be induced by treatment with retinoic acid and dibutyryl cyclic AMP (Bt,cAMP) resulting in morphologically altered parietal endoderm-like cells (3). Therefore, this system has allowed the analysis of the mechanism(s) of gene regulation during the process of cell differentiation.

One such gene whose expression is modulated on differentiation of F9 by treatment with retinoic acid/Bt,cAMP is hst (also referred to as k-FGF or FGF-4). The hst gene was initially discovered through transformation of NIH/3T3 cells with human DNA from various sources (4-6). The Hst protein is a member of the fibroblast growth factor (FGF) family, which also includes acidic FGF (FGF1), basic FGF (FGF2), Int-2 (FGF3), FGF5 (7), FGF6 (8), and KGF (9). Transcription of murine hst has been detected in the preimplantation and early postimplantation embryos (10, 11). In cultured cells, expression of the hst gene is limited to undifferentiated embryonal carcinoma cells including F9. Differentiation of F9 with retinoic acid/Bt,cAMP results in the repression of hst expression (12, 13). The temporal expression of hst gene, therefore, allows us to study transcriptional modulation in molecular details.

Previously, we (14) and others (15) reported that an enhancer element residing in the third exon is essential for positively regulating transcription from the hst promoter in F9. Subsequently, the functional element of this enhancer was delimited to an octamer motif (16, 17). The octamer sequence is known to bind in vitro to both Oct1, a ubiquitous factor, and Oct3, a factor present in undifferentiated embryonal carcinoma cell lines and in early stages of mouse embryo (18). Although the octamer motif plays the role of an enhancer for the hst promoter in F9, to gain a better insight into the mechanisms of hst gene expression in F9, further analysis of the hst promoter region becomes essential. We report here the characterization of a functional NF-Y (CP-1, CBF) (19-21) binding element present in the hst promoter in a segment that we refer to as Y.

EXPERIMENTAL PROCEDURES

Plasmid Constructs and Oligonucleotides—DNA fragments used in this study were derived from the 5'-promoter region of human hst gene. A 277-bp RsaI/Apal (R/A, -172/106) fragment was cloned at the HindIII site in pBluescript KS+, utilizing a HindIII linker. From this fragment, RmaI/SmaI (R/S, -172/-98) was generated and subcloned between HindIII and SmaI sites in pBluescript KS+. The oligonucleotides corresponding to a portion of R/A were synthesized on a DNA synthesizer (ABI, model 380B). Sequences of their sense strands were as follows: Y oligomer corresponds to 5'-CCCCCGGCCCTGATTGGCAGGCGG-3' (-153/-130); Ym with mutation in CCAAT box, 5'-CCCCCGGCCCTCAGGCGG-3'. The other oligonucleotide used in this study was Eco oligomer, 5'-AAATTTTTGATTGGCAGGCGG-3'; present in Eco promoter (19), pY is a 71-bp BamHI/SalI fragment that contains Y oligomer sequence subcloned at the SmaI site of pBluescript KS+.

A mutant hst promoter construct was created by annealing mutagenizing oligonucleotides, Ym and antisense Ym, to R/A cloned at the polylinker primers using either Taq (Promega) or Vent polymerase (New England Biolabs Inc.). The amplified products were annealed and reamplified (double polymerase chain reaction) to give a full-length product between two polylinker primers, digested with HindIII, and cloned in pBluescript KS+. Resulting plasmids were sequenced to confirm mutations, digested with HindIII, and finally cloned upstream of

An Upstream NF-Y-binding Site Is Required for Transcriptional Activation from the hst Promoter in F9 Embryonal Carcinoma Cells*

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‡ The abbreviations used are: Bt,cAMP, dibutyryl cyclic AMP; bp, base pairs; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; FGF, fibroblast growth factor; dF9, differentiated F9.

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the CAT gene in pSVOOCAT as described previously (14). In some CAT constructs, a DNA fragment referred to as 53, which spans 3,548/3,717 of the hst gene and contains an octamer sequence, was inserted at the 3'-end of the CAT gene. RA-OO, Ym-OO, and -64-OO were CAT constructs without fragment 53. RA-53, Ym-53, and -64-53 were those with fragment 53. -64-OO and -64-53 were constructed by the insertion of a polymerase chain reaction-generated -64/92 fragment instead of FUA.

**FIG. 1. Comparison of the human and mouse hst promoters.** Nucleotide sequences of human and mouse hst promoters upstream of transcriptional initiation site (-1) are compared. Stars between the two sequences indicate conserved nucleotides. Dashes have been introduced to maximize homology. A TATA box-like sequence is indicated with a bold line. The dashed line represents the segment Y in which an NF-Y-binding site was characterized in this study. Two restriction sites, Rmal and SmaI, in the human hst promoter region are indicated above the sequence.

DNA-binding Proteins Interact with the Rmal/SmaI Region of the hst Promoter—Fig. 1 shows the promoter regions of human and mouse hst genes. Previously, we narrowed the promoter region of human hst to a 277-bp fragment, RA(-172/105) that, operating concurrently with an octamer element (ATGCAAAT, 3656/3663) residing in the third exon, was sufficient to confer the promoter activity in F9. Furthermore, we observed that deletion of the 5'-region (-172/65) of this promoter substantially decreased its strength suggesting that important regulatory sequences were confined within the deleted fragment of the hst promoter (17).

Since DNA-binding proteins may represent trans-acting factors that affect the transcription of the hst gene, we set out to elucidate the protein that interact with the 5'-end of the promoter of interest. Utilizing the unique SmaI restriction site at position -98, a 75-bp fragment RmaISmaI (R/S; -12/98) was isolated and used as a probe in EMSA.

When the R/S probe was incubated with nuclear extract from F9, a single protein-DNA complex, complex II, was observed (Fig. 2). Nuclear extract from dF9 yielded two additional complexes (I and III). All these complexes were disrupted by the addition of a 200-fold molar excess of unlabeled R/S (lanes 3 and 8) or an oligonucleotide Y (lanes 5 and 10), which was designed on the basis of the DNA contact points that were established for complexes I and II observed with the R/S probe (see below). On the other hand, none of the complex was disrupted when a HinpI/ApiI (32/105) fragment derived from a neighboring region was used as a nonspecific competitor (lanes 4 and 9) or with a mutant Y oligomer, Ym (lanes 6 and 11).
**A Functional NF-Y-binding Site in hst Promoter**

**Fig. 2.** Formation of specific protein-DNA complexes with R/S (-172/-98) probe in EMSA. Binding reactions and electrophoresis were performed as described under "Experimental Procedures." End-labeled R/S fragment from the hst promoter was incubated with 5 μg of nuclear extracts from F9 (lanes 2–6) and dF9 (lanes 7–11). Competitions, both specific (lanes 3, 5, 8, and 10) and nonspecific (lanes 4, 6, 9, and 11), were performed at a 200-fold molar excess. The positions of complexes observed with R/S are shown to the left of the autoradiograms. Lane 1 represents migration of protein-free DNA. NE, nuclear extract; cold, unlabeled competitors; F, free probe.

For complex I (data not shown). These DNA contact points enabled us to recognize a CCAAT box. Complex III did not show any methylated guanine or adenine residue interfering with binding (data not shown).

**The Protein Binding to the CCAAT Box in F9 Nuclear Extract Is NF-Y—**The R/S region of the hst promoter shared a 9-bp perfect homology with the consensus sequence of the Y-box found in the promoters of major histocompatibility complex class II genes (19). Since NF-Y is known to be the nuclear factor that interacts with the Y-box, it became imperative to determine if the nuclear factor(s) binding to the R/S fragment was NF-Y or related factor(s). For this purpose, both strands of an oligonucleotide, Y, which included the sequences pinpointed by methylation interference analysis, along with a mutant oligomer, Ym, were synthesized and annealed, respectively. Also, both strands of an oligonucleotide corresponding to the NF-Y binding site in the Ea promoter were synthesized and annealed. The resulting double-stranded oligonucleotides were then either used as probes or competitors in mobility shift experiments. As can be seen in Fig. 4A, when Y oligomer was used as a probe, a single protein-DNA complex, termed Y, was observed with F9 nuclear extract (Fig. 4A, lane 2). However, when this probe was incubated with nuclear extract from dF9, another protein-DNA complex, YL, migrating with a faster mobility was also observed. The specificity of Y and YL was confirmed by competing with unlabeled wild-type Y oligomer or its mutant version, Ym. As anticipated, while Y oligomer inhibited the formation of both complexes, Ym did not (Fig. 4A, compare lane 5 with lane 3 for F9 and lane 12 with lane 10 for dF9). Next, we wished to see if the formation of Y and YL could also be inhibited by competing with the unlabeled Ea oligomer. At a 200-fold molar excess, like Y, Ea completely inhibited the formation of Y (Fig. 4A, compare lanes 5 with lane 3 for F9 and lane 12 with lane 10 for dF9). However, Ea was unable to inhibit the binding of YL to the same extent as the wild-type Y oligomer (Fig. 4A, compare lanes 10 and 11).

The fact that NF-Y-binding Ea oligomer efficiently disrupted the formation of Y strongly suggested that this complex con-
FIG. 4. Oligonucleotide probe, Y, derived from R/S contains an NF-Y-binding site. A, EMSA was performed with double-stranded Y oligomer probe under the conditions described in the legend to Fig. 2. Sources of nuclear extracts are F9 (lanes 2–8) and dF9 (lanes 8–15). For competitions, Y (lanes 3 and 10), mutant Y (Ym) (lanes 5 and 12), and Eo (lanes 4 and 11) oligomers were included. For supershift experiments, 0.6 μl of YA1a monoclonal antibody against NF-YA (lanes 6 and 13), and 0.06 μl of an affinity-purified polyclonal antibody specific for NF-YB (lanes 7 and 14) or a 1:20 dilution of antiserum also specific for NF-YB (lanes 8 and 15) were used. B, for supershift experiments, 0.1 μl of affinity-purified polyclonal antibody specific for NF-YB (lanes 1, 3, 5, and 7) or a 1:10 dilution of antisera (lanes 2, 4, 6, and 8) was used. Y or Eo probes are shown above the respective lanes. C, binding of NF-Y to its cognate binding site in Eo oligomer probe. End-labeled Eo probe was incubated with nuclear extracts from F9 (lanes 2–7) and dF9 (lanes 8–13), and complexes were competed or supershifted as described in A (above). Lane 1 in panels A and C represents migration of protein-free DNA. Position of the supershifted complex is shown by an arrow in panel A, and positions of YU, NF-Y, and YL are shown to the left of the panels. NF, nuclear extract; cold, unlabeled competitor; F, free probe; Ab, antibody; YA1a, monoclonal antibody against NF-YA; A, P, and r.s., affinity-purified polyclonal antibody and rabbit serum against NF-YB, respectively.

sisted of NF-Y. In order to establish this, monoclonal and polyclonal antibodies against NF-Y were included in the reaction mixtures before W and YL were separated by EMSA. The clonal antibodies against NF-Y were included in the reaction mixture before W and YL were separated by EMSA. The monoclonal antibody YA1a, which binds to the glutamine-rich activation domain of the NF-YA subunit, supershifted W (Fig. 4A, lanes 6 and 13), and had no influence on YL (Fig. 4A, lane 13). Again, at appropriate dilutions, addition of affinity-purified polyclonal anti-NF-YB or anti-NF-YB rabbit serum to the reaction mixtures inhibited the formation of YU and primarily led to aggregated material at the top of the gel (Fig. 4B, lanes 1–4). In contrast, the binding or mobility of YL was not affected (Fig. 4B, lanes 3 and 4). As a control, a similar mobility shift assay was performed with the Eo oligomer as the probe. As can be seen in Fig. 4C and Fig. 4B, lanes 5–8, results similar to those obtained with the Y oligomer probe were observed except that a complex equivalent to YL was not observed with nuclear extract from dF9 (compare Fig. 4C, lane 8 with Fig. 4A, lane 9). These results demonstrate that YU is NF-Y and suggest that YL may represent an independent DNA-binding protein.

We also employed UV-induced cross-linking experiments to determine the polypeptide compositions of YU and YL. YU yielded three cross-linked polypeptide bands with mobilities corresponding to approximately 47, 45, and 37 kDa when this complex was resolved on a denaturing SDS-polyacrylamide gel (Fig. 5, lanes 3, 5, and 7). The mobilities of these bands were similar to those reported for the cross-linked subunits of NF-Y (28). YL, on the other hand, yielded a single cross-linked polypeptide band with a mobility of approximately 70–80 kDa (lanes 2 and 4). An approximately 200 kDa band in lane 2 was not reproducible and probably represented an experimental artifact.

hst Promoter Activity Is Attributable to the NF-Y-binding Site—In order to evaluate the functional significance of the NF-Y-binding site that we characterized, CAT reporter plasmids having mutations in the CCAAT core sequence of the hst promoter were constructed. These promoter constructs were transfected into F9, and their CAT activities were compared with the level obtained in parallel transfections with the parent, RA-53 (Fig. 6, lane 4), to give a measure of the promoter activity. Mutation in the CCAAT sequence of the NF-Y-binding site in segment Y (Ym-53) reduced the promoter activity by about 70% (lane 2). Deletion of Y (~64–53), as expected, brought the promoter activity to the basal level (lane 6). These results clearly demonstrate that the NF-Y-binding site in segment Y of the hst promoter and the octamer element present in the noncoding region of the third exon are the essential activating elements of the hst gene.

NF-Y Associates with a Differentiation-induced Nuclear Factor—The implication of NF-Y as a nuclear factor that positively regulates the expression from the hst promoter in F9 led us to hypothesize that in differentiated cell lines a differentiation-induced nuclear factor may associate with NF-Y. This assumption was tempting for two reasons. First, methylation interference results had shown that the DNA contact points for complex I were identical to those established for NF-Y (data not shown). Second, the intensity of NF-Y (complex II) from the dF9 nuclear extract was conspicuously lower than that observed with nuclear extract from F9, although equal amounts of nuclear extracts from both F9 and dF9 were used (Fig. 2, compare lane 2 with lane 7). To test this hypothesis, the R/S probe was incubated with dF9 nuclear extract in the presence of the monoclonal antibody YA1a. As can be seen in Fig. 7A, complex I, like NF-Y (complex II), was supershifted. We then considered the possibility that the flanking sequences may be required for stabilization of complex I in vitro since unlabeled Y oligomer efficiently disrupted complex I, but when used as a
A Functional NF-Y-binding Site in hst Promoter

**Fig. 5.** UV cross-linking of nuclear proteins from F9, dF9, and Jurkat. UV irradiated YU and YL complexes were separated by EMSA, cut from the gel, and analyzed on SDS-polyacrylamide gel electrophoresis. Lane 1 is a sample derived from a portion of the EMSA gel unrelated to complex. Lanes 3, 5, and 7 represent YU from Jurkat, dF9, and F9, respectively; lanes 2 and 4 represent YL from Jurkat and dF9, respectively. In lane 6, a faint UV cross-linked complex (supposedly YL from F9 nuclear extract) was analyzed. Molecular mass markers are indicated by arrows. Open arrowheads indicate the position of cross-linked complexes.

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<th>Cell</th>
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**Fig. 6.** NF-Y-binding site is required to stimulate transcription from the hst promoter. CAT activities of a series of hst-CAT expression constructs transfected into F9 were determined as described under "Experimental Procedures." The thin layer chromatogram shown is acetylation of [dichloroacetyl-14C]chloramphenicol with 40 µl of cellular extracts of transiently transfected F9. Relative CAT activities are the values corrected for transfection efficiencies by expression of cotransfected pRSV-LacZ. 53 represents a 3'-fragment of the hst gene that contains the octamer sequence. The cross in Y represents mutations in this segment. Each transfection was performed as four independent experiments, and the chromatogram represents a typical transfection.

probe it did not yield this complex (Fig. 4A). To test this possibility, a 41-mer oligomer bearing the core CCAAT sequence (−159/−119) was used as a probe in EMSA; still this complex did not appear (data not shown). Then we designed a probe, pY, in which the Y oligomer was flanked with sequences derived from the pBluescript KS+ plasmid (see "Experimental Procedures"). When this probe was incubated with nuclear extracts from F9 or dF9, protein-DNA complexes similar to those established earlier with the R/S probe (Figs. 2 and 7A) were seen (Fig. 7B). Complex I, in addition to being inhibited by competing with Y and Ea oligomers (Fig. 7B, lanes 8 and 9), was again supershifted with YAla (lane 11). From these results we conclude that, in complex I, NF-Y is associated with a differentiation-induced nuclear factor and that this interaction most likely involves a protein-protein interaction between NF-Y and a non-DNA-binding protein.

**DISCUSSION**

Although the enhancer activity of an octamer element residing in the noncoding region of the third exon of hst has been demonstrated (14–16,29), the mechanism(s) of hst activation in F9 has not been established as yet. In the present study, we have characterized the functional region of the hst promoter.

The sequence harboring the CCAAT box in hst promoter shared a perfect homology with 9 bp of the CCAAT-bearing consensus Y-box sequence found in the promoters of major
NF-Y, in dF9, is complexed with a differentiation-induced nuclear factor. EMSA and supershift experiments were performed as described in the legends to Figs. 2 and 4, respectively, with end-labeled R/S as probe (A) and with a 71-bp pY probe (B) (see text). Source of nuclear extracts and competitors is indicated above the lanes. Arrowheads represent the position of complex I and arrows the position of supershifted complexes. YAla, monoclonal antibody against NF-Y. Abbreviations are explained in the legend to Fig. 4.

histocompatibility complex class II genes (19). NF-Y, which interacts with the Y-box, is required not only for the accurate and efficient transcription of major histocompatibility complex class II genes but also for the transcription from several promoters such as the rat albumin (24,30) and α collagen promoters (31). Here, we detected a major protein-DNA complex, W, binding with high specificity to Y oligomer spanning -1531-130 of the hst promoter. Formation of W was completely inhibited by competing with the NF-Y-binding Ea oligomer (Fig. 4A). More importantly, monoclonal antibody YAla supershifted this complex but had no influence on YL, confirming the specificity of this supershift (Fig. 7A). Also, affinity-purified polyclonal anti-NF-YB and anti-NF-YA serum prevented the formation of this complex (Fig. 4, A and B). Furthermore, three cross-linked polypeptides observed for YU (Fig. 5) are in agreement with those reported earlier for NF-Y (28). These data strongly suggest that NF-Y is associated with a differentiation-induced nuclear factor and suggest that this association may involve a protein-protein interaction between NF-Y and a non-DNA-binding protein.

YL, observed with the Y oligomer probe, also appears to be a differentiation-induced nuclear factor for the following reasons. First, it competed in a sequence-specific manner (for example compare lane 12 with lane 10 in Fig. 4A). Second, a UV-induced cross-linking of this complex yielded a single polypeptide band with a mobility of approximately 70–80 kDa (Fig. 5). Third, when F9 was induced to differentiate with retinoic acid/Bt',cAMP, YL appeared after 2 days of differentiation. Attempts to purify these factors are in progress and will help us to elaborate further.

The results presented in this report have demonstrated that NF-Y motif is an essential activating element of the hst promoter in F9. The residual activity observed with either the intact NF-Y or the octamer binding site alone is below 30% of the response with both intact promoter and 3'-enhancer element (Fig. 6). This line of evidence suggests that a concerted action of these elements and their respective transcription factors is required for maximum induction of hst gene expression.

3 S. Hasan, unpublished data.
The differential expression of Oct3 in F9 may lead one to hypothesize that it could primarily be responsible for modulating the expression of hst in F9. However, we have shown that the expression of oct3 cDNA in HeLa or PYS-2 cells, which do not express hst, did not rescue the expression from the hst promoter (17). Similarly, Shimazaki et al. (32) have shown that transfection of Oct3 expression plasmid in HeLa cells did not rescue a P19 (embryonal carcinoma cell) specific enhancer. Oct3 has also been shown to play a suppressive role in F9 (33). These lines of evidence indirectly suggest the involvement of additional factor(s) that may selectively recruit Oct1/Oct3 to interact with NF-Y or bridge these with the general transcriptional machinery of the hst in F9. Such a mechanism has been demonstrated for differential promoter activation by Oct1 through selective corecruitment with herpesvirus trans-activator VP16 (34).

Alternatively, a protein-protein interaction between a differentiation-induced nuclear factor and NF-Y may mask the transcriptional activation domain of NF-Y without altering its DNA binding activity in a manner analogous to the binding of GAL80, a negative regulator, to the transcriptional activation domain of GAL4 (35). Work is in progress to characterize the nuclear factor that associates with NF-Y. This will help in elaborating on the possible mechanisms by which transcription of the hst gene is modulated.

In summary, we have demonstrated that, in addition to a distal octamer enhancer element, a functional NF-Y binding site in the proximal promoter region is required for full activation of the hst gene in F9. Our study now provides a useful model that may enable one to study the roles of NF-Y- and octamer-binding proteins in developmental regulation.

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