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Regulatory sequences required for *hst-1* expression in embryonal carcinoma cells

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Abstract

The *hst-1* gene, which is implicated in mammalian embryonic development and morphological transformation of NIH3T3 cells, is expressed in undifferentiated F9 cells, but not in differentiated F9 and other well-differentiated cells, such as PYS-2, NIH3T3 and HeLa cells. An octamer element present in the 3′ untranslated region acts as an enhancer. Although Oct3 is down-regulated when F9 cells are differentiated, transient expression of Oct3 did not enhance the *hst-1* promoter activity in HeLa, NIH3T3 or PYS-2 cells. Thus, the role of Oct3 on *hst-1* expression remains elusive, and an additional transcription factor which interacts may regulate *hst-1* transcription in association with Oct1, Oct3 or both.

Key words: hst-1/K-FGF/FGF4; *oct-3*; Enhancer; F9; Differentiation

1. Introduction

The *hst-1* gene (*K-FGF/FGF4*) is a transforming gene initially identified through transfection of high molecular weight DNA from various sources into NIH3T3 cells [1–3]. Hst1 protein is homologous to members of the fibroblast growth factor (FGF) family [4,5]. Among 7 members of this group,*int-2/FGF3* [6–8], *FGF5* [9], and *hst-2/FGF6* [10–12], in addition to *hst-1* [13–16] are exclusively expressed in embryo and embryonal carcinoma cells; acidic and basic FGFs are expressed both in embryonal carcinoma cells and in differentiated cells [15]; and *KGF* is expressed in keratinocytes [17]. During the embryonic development of mouse, *hst-1* is expressed as early as the late blastocyst stage and its expression becomes restricted to the tail bud which is the primary source of the mesoderm [18]. The *hst-1* gene is also expressed transiently in the branchial arc units, the somitic myotome, the limb and tooth buds, and thus may have multiple roles in embryonal development [18]. Transcription of *hst-1* is turned off when F9 cells differentiate into endoderm-like cells [13,14]. The transcription is dependent on the non-coding region of the third exon [19–22]. Octamer-like [23] and engrailed (En)-like [24] sequences present in the third exon have been considered as the candidates. Shoorlemmer and Kruijer [22] reported the involvement of the octamer motif by deletion experiments.

Oct3, which is expressed in undifferentiated embryonal carcinoma cells and is down-regulated after differentiation, may regulate *hst-1* expression. In fact Ma et al. [21] reported that transfection of an *oct-3* expression plasmid into PYS-2 cells enhanced *hst-1* promoter activity. However, the proof that the *hst-1* gene is the target for Oct3 has not yet been unambiguously established. Here we report the mutational analysis of the 3′ enhancer region of the human *hst-1* gene and the effect of the transfected *oct-3* expression plasmid on the *hst-1* promoter in various differentiated cells. Our results indicate the involvement of an additional transcription factor which is expressed differentially in F9 cells and which interacts with either Oct1, Oct3 or both.

2. Materials and methods

2.1. Plasmids and synthetic oligonucleotides

A human *hst-1* clone, BM5, which was isolated from an NIH3T3 transformant [2], was used to construct CAT plasmids. The sequence was numbered such that the major transcriptional initiation site was +1 which corresponds to 2,279 of the human genomic *hst-1* reported by Yoshida et al. [25]. To a unique *Hin*dIII site of pSVOOCAT [26], the following DNA fragments derived from BM5 were inserted using an *Hin*dIII linker: MA, a *Mbo*I–*Apa*I fragment spanning from 1,295 to 105; BA, a *Bsr*I–*Apa*I fragment spanning from −282 to 105; RA, a *Rma*I–*Apa*I fragment spanning from −172 to 105; −64/92, a DNA fragment spanning from −64 to 92 generated by polymerase chain reaction (PCR) [27] between a sense (5′ctaaagcttGGAGCGGGCGAGTAGGA3′) and an antisense (5′agtaagcttCTCTACCCCGGCTGCAT3′) primer. The *Bam*HI site of pSVOOCAT was converted to an *Xba*I site where the following DNA fragments were inserted. E2S, a *Bst*E2–*Sal*I fragment spanning from 3,318 to 3,881; 51, a DNA fragment spanning from 3,193 to 3,392 generated by PCR between a sense (5′atatctAGATAGCGTCGGTGCGTCC3′) and an antisense (5′ggatctagaCTTCCACGGTTGCTTCA3′) primer; 52, a DNA fragment spanning from 3,376 to 3,517 generated by PCR between a sense

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(5'aggtctagaTGAAGCAACCGTGGAAG3') and an antisense (5'cagtctagaGAAACATTCGGAGCATT3') primer; 53, a DNA fragment spanning from 3,548 to 3,717 generated by PCR between a sense (5'gtgtctagaTCTGAACTGTCTCCCGA3') and an antisense (5'ccgtctagaGCAAGTGTCTTCTTTCT3') primer; and 52+53, a DNA fragment spanning from 3,376 to 3,717 generated by PCR between a sense (5'aggtctagaTGAAGCAACCGTGGAAG3') and an antisense (5'ccgtctagaGCAAGTGTCTTCTTTCT3') primer. 53M1,53M2 and 53M3 were mutant 53 fragments produced by site-directed mutagensis using 5'GTTCGAATGCcccTCATCGACTT3' and 5'TCATCGACTTccc-ATACTATTCT3' oligonucleotides for mutating octamer/En-like sequences (see Fig. 1C). These mutant 53 fragments were cloned into pUC18, sequenced, cut with *Xba*I and inserted into the *Xba*I site of CAT plasmids. A 39mer dsDNA fragment spanning 3,650–3,688 (E39) was produced by annealing two synthetic oligonucleotides. A 22 bp ds-oligonucleotide, the sense strand of which was 5'TGTCGAATGC-AAATCACTAGAA3', containing a consensus octamer sequence but not an En-like sequence, was synthesized. A cytomegalovirus-promoter(CMV) driven *oct-3* expression plasmid was supplied by Dr. H. Hamada [28].

2.2. Cell culture and CAT assay

F9 cells were cultured in gelatin-coated dishes and HeLa, NIH3T3 and PYS-2 cells were cultured in conventional plastic dishes 100 mm in diameter. For transfection with various plasmids, a total of 30 or 31 μg DNA (by adding pUC18 DNA) was co-precipitated with calcium phosphate as previously described [20]. Cells were exposed to calcium precipitates for 24 h and harvested 24 h thereafter. The CAT assay was performed as described previously [20] and, in several experiments, the activity was corrected for transfection efficiency by expression of co-transfected pCH110 [29] or pRSV-LacZ plasmid. The latter was constructed by insertion of a RSV-driven *lacZ* gene into pBluescript KS (T.K., unpublished). For differentiation of F9, 1 mg/ml dibutyryl cyclic AMP and 10^{-6} M retinoic acid were added at day −2 of the transfection.

2.3. Band shift assay

Nuclear extracts were prepared as described by Schreiber et al. [30], adding the following protease inhibitors; 1 μM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A and 0.3 μg/ml antipain. Probes were 5' end-labeled with [γ-^{32}P]ATP. Binding reactions were carried out in 20 μl solutions containing 20 mM Tris-HCl (pH 7.8),50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT and 5% glycerol [31]. 10,000 cpm of probe (containing 0.1–0.5 ng) and 5 μg of nuclear extract were incubated at room temperature for 10 min. To reduce the non- specific binding of nuclear proteins to the probe, 2 or 4 μg of poly(dI-dC) were included in the mixture. 5 μl fractions of the reaction mixtures were resolved by 4% polyacrylamide gel electrophoresis [32]. For competitive band shift assays, a 200-fold molar excess of unlabelled DNA fragments or equivalent amounts of pUC18 were used.

3. Results

As E2S (3,318/3,881) showed enhancer activity when inserted 3' to MA-OO-CAT [20], we tested the enhancer activity of the PCR-generated fragments, 51, 52, 53 and 52 + 53. Fragments 53 and 52 + 53 enhanced the *hst-1* promoter almost 10 fold (Fig. 2A and data not shown). In 53, an octamer sequence, ATGCAAAT (3,656/3,663),and an En-like sequence, ACTTAAAATA (3,669/3,678) are present, although the octamer sequence in the mouse was ATGCtAAT. We next tried to discriminate between the enhancer activity of these two motifs. Three mutant fragments, 53M1, 53M2 and 53M3, were generated, replacing the AAA nucleotides in 53 by CCC; then they were inserted downstream of MA-OO-CAT (Fig. 1). In F9 cells, 53M1 showed 4-fold less promoter activity

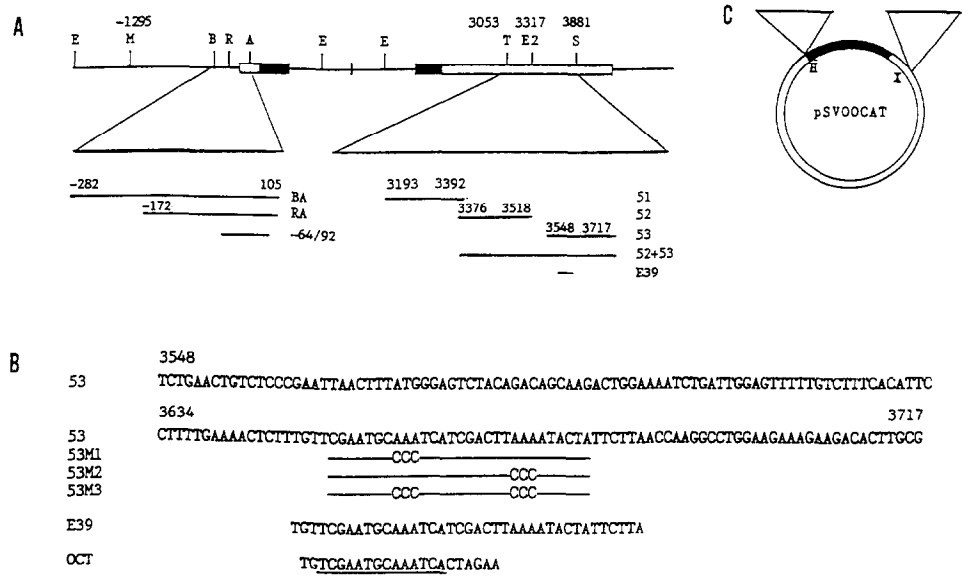

Fig. 1. DNA fragments and CAT constructs used in this study. (A) A restriction map of genomic *hst-1* and the DNA fragments derived from it. On the top, restriction sites and exon/intron organization of the human hst-1 gene are shown. Filled and open boxes represent coding and non-coding portions of the transcribed sequence, respectively. The restriction sites, *Apa*I, *Bsr*I, *Bst*E2, *Eco*RI, *Eco*T22I, *Mbo*I, *Rma*I and *Sal*I are abbreviated as A, B,E2, E, T,M, R and S, respectively. (B) The sequence of fragment 53 and partial sequences of mutated 53. The sequence of the 53 mutants are the same as that of wild-type 53, except for replacing the AAA residues by CCC. E39 is a synthetic dsDNA which contains both octamer and En-like sequences and OCT is a 22mer ds-oligonucleotide containing an octamer element. (C) Structure of CAT constructs. The bacterial CAT gene is shown as a thick black arc, and the sequences derived from SV40 and pBR322 as an unfilled arc. DNA fragments tested for promoter activity were inserted at the *Hin*dIII site (H). DNA fragments tested for enhancer activity were inserted at the *Bam*HI site after converting to an *Xba*I site (X).

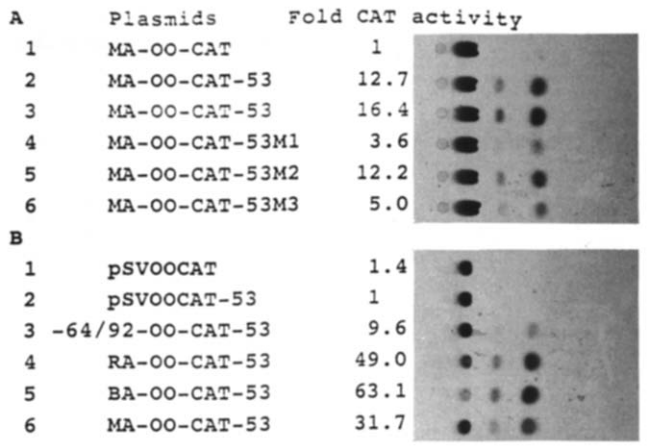

Fig. 2. (A) Enhancer activity of wild-type and mutant 53. Cells were transfected with 20 μg of CAT constructs, 10 μg of pUC18 and 1 μg of pCH110. F9 nuclear extracts corresponding to 30 μU of β-galactosidase activity were assayed for CAT activity. One out of three experiments is shown and the results were reproducible. (B) Promoter activity of the 5′ region of human *hst-1*. Transfection protocols were the same as A except that pRSV-LacZ was used in place of pCH110. F9 nuclear proteins corresponding to 7.5 μU were assayed for CAT activity. One out of three experiments is shown and the results were reproducible.

than wild-type 53, whereas the M2 mutation did not reduce the activity (Fig. 2A), implying that the octamer, but not the En-like sequence, is active as an enhancer.

The transcription of *hst-1* is repressed in F9 cells after differentiation [13,14]. Expression of Oct3 is also down-regulated after differentiation [33]. Oct3 protein is found only in undifferentiated embryonal carcinoma cells and at the early stage of embryogenesis [28,34,35]. In order to determine whether or not the octamer and En-like sequences of 53 bind Oct1 and/or Oct3 proteins in F9 nuclear extract, we performed band shift assays. Probe 53 detected Oct1 and Oct3 proteins in F9 nuclear extract (Fig. 3A, lane 3) but the probe detected only Oct1 protein in nuclear extracts of HeLa and NIH3T3 cells (data not shown). The nuclear extract of HeLa and NIH3T3 cells which were transiently transfected with an *oct-3* expression plasmid yielded authentic Oct3 bands (see Fig. 4). In order to further confirm that the shifted bands actually bound the octamer proteins, a ds synthetic 22mer oligonucleotide containing the ATGCAAAT core sequence was used as a competitor in the band shift assay. The synthetic 22mer competed out both Oct-1 and Oct-3 proteins in F9 cells (Fig. 3A, lane 2). The mutant 53 probes were also tested by band shift assay. Wild-type 53 and 53M2 probes detected Oct1 and Oct3 proteins in F9 cells (Fig. 3A, lanes 3 and 5), but 53M1 and 53M3 probes detected only small amounts, if any, of Oct1 and Oct3 proteins (lanes 4 and 6). In competitive band shift assays, wild-type 53 ad 53M2 almost completely competed out the binding of labeled E39 probe to Oct1 and Oct3 proteins of F9 nuclear extracts (Fig. 3B, lanes 2 and 4).

In order to define the promoter region of the *hst-1* gene, 5′ fragments shorter than MA were tested for promoter activity. Two DNA fragments, −172/105 (RA) and −282/105 (BA) had promoter activity substantially higher than MA, whereas −64/92 retained weak activity when these fragments were inserted upstream of the CAT gene of pSVOOCAT-53 (Fig. 2B). Ma et al. [21] observed positive regulatory activity between −267/−61 of the mouse *hst-1* 5′ sequence which corresponds to −298/−65 of the human counterpart. In this study, we narrowed the promoter-active region to −172/−65.

To test whether or not Oct3 directly enhances the *hst-1* promoter, a CMV-driven *oct-3* expression plasmid [28] was co-transfected into HeLa, PYS-2 and NIH3T3 cells with various CAT constructs. The *oct-3* expression plasmid was sequenced again before this experiment and its authenticity was verified. The transfected *oct-3* plasmid was expressed, at least in HeLa and NIH3T3 cells, as judged by the mobility shift experiment (Fig. 4, lanes 2, 4, 6, 14, 16 and 18). Such shifts which appeared below *oct-3* of *oct-3*-transfected HeLa cells (lanes 2, 4 and 6) were not demonstrable when the 22mer octamer probe was used instead of E39. However, in PYS-2, nuclear extracts which had been co-transfected with the *oct-3* expression plasmid showed very weak Oct3 signals

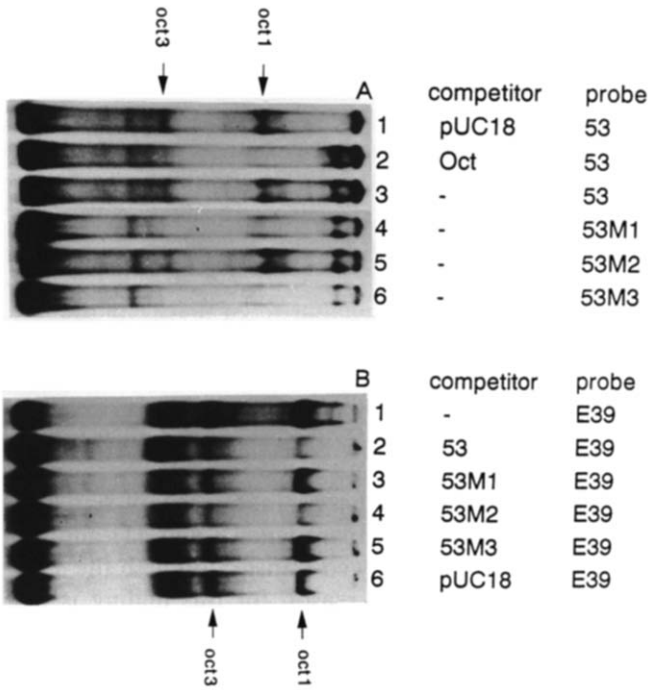

Fig. 3. (A) Band shift of wild-type and mutant 53 probes by undifferentiated F9 nuclear extract. The following probes were used: wild-type 53 (lanes 1–3); 53M1 (lane 4); 53M2 (lane 5); 53M3 (lane 6). Competitors were a 200-fold molar excess 22mer oligonucleotide containing only the octamer sequence (lane 2) or 10 ng of pUC18 (lane 1). (B) Competition of band shifts by wild-type and mutant 53. Band shifts were performed using F9 nuclear extract and labeled E39 oligonucleotide, in which a 200 molar excess of competitors were included. The competitors used were: no competitor (lane 1); wild-type 53 (lane 2); 53M1 (lane 3); 53M2 (lane 4); 53M3 (lane 5); and 260 ng pUC18 (lane 6).

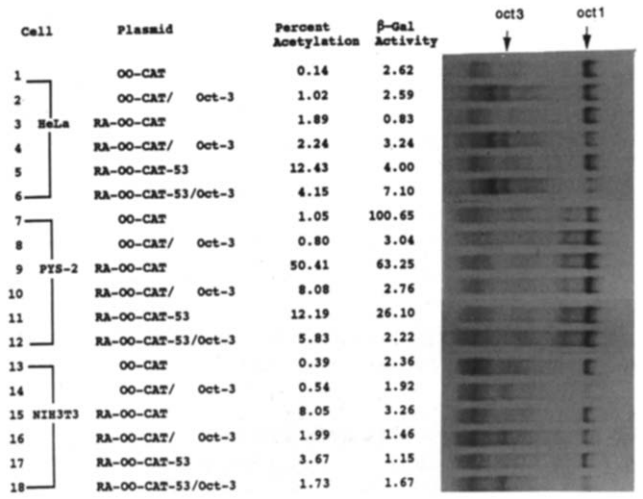

Fig. 4. Transient expression of Oct3 did not enhance *hst-1* promoter activity in HeLa, PYS-2 and NIH3T3 cells. Transfected calcium precipitates contained 20 μg CAT constructs, 3 μg CMV-oct-3 and 1 μg pRSV-LacZ. Lanes 1–6, HeLa cells; lanes 7–12, PYS-2 cells; and lanes 13–18, NIH3T3 cells. Transfected plasmids were as follows:OO-CAT (lanes 1, 7 and 13); OO-CAT plus *oct-3* (lanes 2, 8 and 14); RA-OO-CAT (lanes 3, 9 and 15); RA-OO-CAT plus *oct-3* (lanes 4, 10 and 16); RA-OO-CAT-53 (lanes 5, 11 and 17); RA-OO-CAT-53 plus *oct-3* (lanes 6, 12 and 18). CAT activities represent the gross percentage of chloramphenicol acetylation for 3 h at 30°C. β-Galactosidase activity was expressed as μU per μl nuclear extract. For the band shift assay, E39 was used as the probe. One out of three experiments is shown. The results of other experiments were comparable to those shown here.

(Fig. 4, lanes 8, 10 and 12). CAT activity in these experiments were not corrected for β-galactosidase activity of co- transfected pRSV-LacZ, since transfection of *oct-3* expression plasmid reduced β-galactosidase activity of PYS-2 cells consistently (Fig. 4, lanes 8, 10 and 12). The results of Fig. 4 clearly demonstrated that Oct3 did not enhance the reporter RA-OO-CAT-53. We also co-transfected the CMV-driven *oct-3* expression plasmid with reporter genes into F9 cells undergoing differentiation, but no enhancement of RA-OO-CAT-53 and BA-OO-CAT-53 was observed (data not shown). In PYS-2 cells RA-OO-CAT showed higher promoter activity than RA-OO-CAT-53 (Fig. 4, compare lanes 9 and 11) and the activities were reduced by co-transfection with the *oct-3* expression plasmid. The reason for this finding is not clear at present, but is repeatedly observed.

4. Discussion

Previous reports have suggested that the enhancer activity of the octamer motif is present at the 3′ end of human [19,20,22] and mouse [20,21] *hst-1* genes. Among these investigations the most thorough was that of Schoorlemmer and Kruijer [22], which demonstrated that the deletion of the candidate octamer sequence resulted in the loss of enhancer activity. In this investigation, we confirmed their finding and demonstrated that the octamer sequence present at 3,655, but not the En-like sequence present near-by had enhancer activity.

Tissue specific gene activation through two octamer binding proteins, Pit1 and Oct1, have been analyzed in detail [36]. In the immunoglobulin heavy chain gene, two octamer sequences are present both 3′ and 5′ to the coding region [37,38]. The *hst-1* gene may be unique in that the tissue specific transcription is enhanced by the octamer sequence present only 3′ to the coding region. The location of the octamer sequence 3′ to the coding region is not unique to *hst-1*, but is rather common to the genes for members of the *FGF* family such as *KGF*[17] and basic *FGF* [38]. The role of these octamer motifs has not been analyzed as yet.

As Oct3 expression is down-regulated along with *hst-1* when F9 cells differentiate to parietal endoderm-like cells, it is conceivable that Oct3 activates the *hst-1* promoter. In fact, Ma et al. reported that transfection of a CMV-driven *oct-3* expression plasmid into differentiated F9 cells and PYS-2, a cell line having characteristics similar to differentiated F9, resulted in the activation of the murine *hst-1* promoter [21]. However, in this study, transient expression of Oct3 did not enhance transcription of an *hst-1*-CAT-octamer reporter gene in either PYS-2, HeLa or NIH3T3 cells (Fig. 4). We also failed to demonstrate the enhancement of the same reporter gene by co-transfection of the *oct-3* expression plasmid into differentiated F9 cells (data not shown). In these experiments, the CAT activities were not corrected for β-galactosidase activity, since expression of Oct3 protein down-regulated the promoter activity [39,40]. In the present study, when the *oct-3* expression plasmid was introduced into PYS-2 cells, β-galactosidase activities were reduced dramatically (Fig. 4). Such a difference between *oct-3*-transfected and untransfected PYS-2 cells might not be ascribed to difference in transfection efficiencies. Therefore, we are afraid that correction of the CAT activity by transfection efficiency using the β-galactosidase activities of co-transfected pCH110 or pRSV-LacZ may be misleading in these experiments.

The reason for the lack of activation of *hst-1* promoter by Oct3 may be the absence of a transcription factor in differentiated cells such as HeLa, PYS-2 and NIH3T3 cells. Alternatively, a transcriptional repressor may be absent in undifferentiated F9 cells, thus allowing the octamer binding factor to interact with the promoter factors. Such a mechanism has been reported for the transcriptional repression of HIV by the protein factor, LBP-1 [41]. We are currently analyzing the protein factors which bind to the promoter region of *hst-1*.

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