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Regulation of Human *hst* Expression by an Enhancer Element Residing in the Third Exon

Akio Sasaki,[1] Mitsumasa Kubo, Shahid Hasan, Yoko Yano and Mitsuaki Kakinuma

Section of Bacterial Infection, Institute of Immunological Science, Hokkaido University, Kita-15, Nishi-7, Kita-ku, Sapporo 060

The *hst* gene is exclusively expressed in undifferentiated embryonal carcinoma cell lines and at a limited stage of embryonal development. Two DNase I-hypersensitive sites were mapped in the 3′ region (approximately 3.5 and 4.5 kb downstream of the translational initiation site) of the human *hst* gene, irrespective of the presence or absence of *hst* mRNA in the cells. A DNA fragment containing one of these DNase I-hypersensitive sites (at around 3.5 kb relative to the translational initiation site) showed enhancer activity when tested by chloramphenicol acetyltransferase (CAT) assay. These results strongly suggest that an enhancer element(s) exists in the third exon of the *hst* and that the expression of the *hst* may be regulated by the presence or absence of a putative protein factor(s) which binds to the enhancer.

Key words: *hst* — CAT assay — DNase I hypersensitivity

The human *hst* gene (*HST1*,) was originally identified through transformation of NIH/3T3 with DNA from human stomach cancers.[1] Since then, a number of laboratories have found *hst* in the same manner.[2–6] The mouse homologue of *hst* is also known.[7] The *hst* encodes a polypeptide chain[5, 8] that shows partial homology with members of the fibroblast growth factor (FGF) family; basic FGF, acidic FGF, int-2, FGF5, hst2/FGF6 and KGF.[9–12] *hst* and *int-2* are closely linked in human chromosome 11q13[13, 14] as well as in the mouse.[7] In the mouse, *hst* is exclusively expressed in embryonal testis and in embryonal carcinoma cell lines, notably in F9.[15, 16] Similarly, in humans, the expression is limited to teratomas[17] and embryonal carcinoma cell lines such as NCC-IT[17] and NT2/D1.[18] *hst* and *int-2* are differentially transcribed in F9; undifferentiated F9 expresses *hst*, while after differentiation with retinoic acid/cyclic AMP, *hst* expression is switched off, but the expression of *int-2* commences.[15, 16] As hst protein contains a signal peptide in its N-terminal portion[8] and as antibody against hst protein reversed the transformation phenotype of NIH/3T3 transfected with the *hst*,[5] it is likely that, in the transformants, hst protein is synthesized and secreted from the cell, and stimulates the cell through as-yet-uncharacterized hst receptors by a typical autocrine mechanism.[19]

Previously, we cloned the human genomic *hst* from two sources; one (BM5) from an NIH/3T3 transformant cell line,[2] and the other (SU7) from a placental library (unpublished). These two *hst* clones have essentially identical DNA sequences and these sequences were also identical to that of an apparently normal *hst* clone derived from a human leukemia cell studied by Yoshida *et al.*[3] The base changes from SU7 to BM5 were as follows: T to C at 149 in the 5′ untranslated region of the exon 1; C to G and T to C, at 926 and 966, respectively, in the first intron; A to G, T to G, C to A and A to T, at 2,087, 2,410, 2464 and 2,524, respectively, in the noncoding region of the third exon. The sequence was numbered relative to the translational initiation site which was assigned by a primer extension analysis (unpublished data). One of the base changes, at position 2,087, was apparently polymorphic among human individuals (unpublished results). There normal *hst* clones transformed NIH/3T3 with the same efficiency as that of a transformant-derived *hst* clone, BM5 (references 3, 6 and unpublished data). These findings suggested that the transformation of NIH/3T3 by *hst* was dependent on the transcriptional activation of the *hst* integrated in the chromosome of NIT/3T3.

In this study, we searched for the transcription-regulatory region which is active in embryonal carcinoma cells but not in NIH/3T3. DNase I hypersensitivity test and CAT assay revealed that a candidate for an embryonal cell-specific enhancer resided in the untranslated region of the third exon.

For the DNase I hypersensitivity experiment, nuclei were isolated from NCC-IT[20] and digested with various concentrations of DNase I for the time periods indicated in Fig. 2. After DNA extraction and DNA digestion with either *Taq*I (for the 5′ region) or *Eco*RI (for the 3′ region), Southern blot hybridization was carried out

[1] To whom correspondence should be addressed.

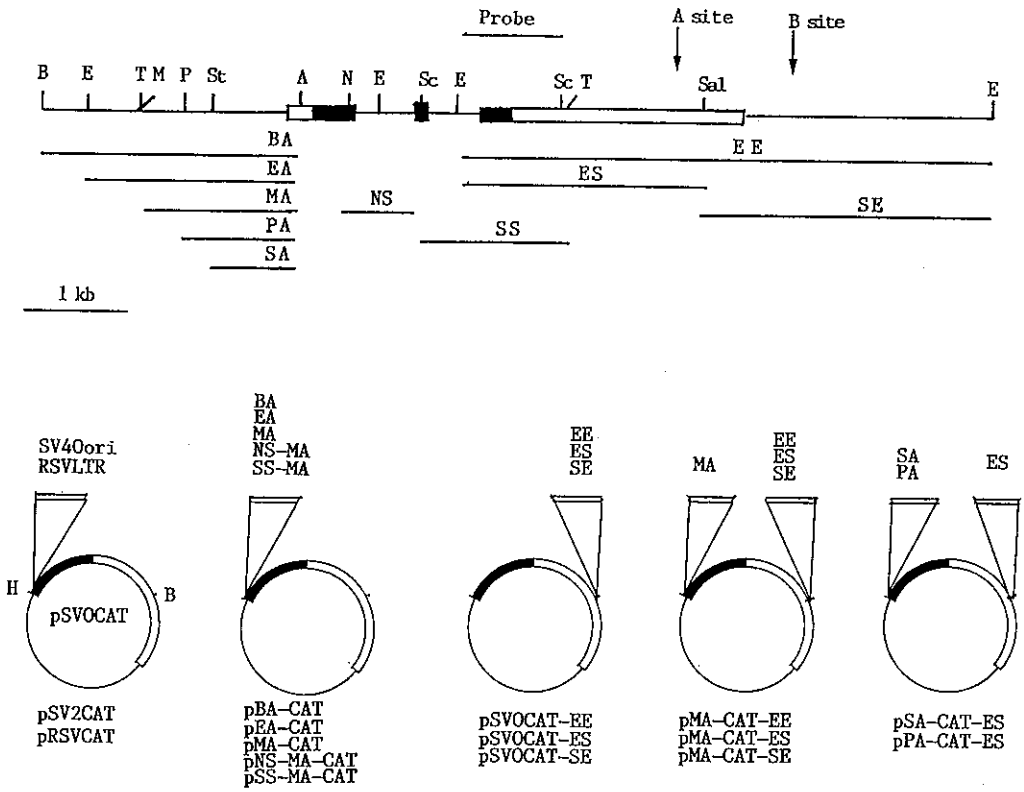

Fig. 1. The organization of human *hst* region, its fragments and construction of CAT reporter plasmids are shown. The upper panel depicts the human genomic *hst* region and its DNA fragments. Open boxes indicate noncoding exons and dark boxes coding sequences. An *Eco*RI/*Taq*I fragment was used as a probe for DNase I hypersensitivity experiment. Sites A and B show DNase I-hypersensitive sites (see Fig. 2). The lower panel shows CAT constructs. Open boxes represent DNA fragments inserted in pSVOCAT. The open arc, thick black arc and thin line of pSVOCAT represent SV40-derived sequence, CAT gene and pBR322-derived sequence, respectively. CAT plasmids were constructed in the following way. For insertion at the *Hin*dIII site upstream of the CAT gene, DNA fragments were ligated with *Hin*dIII. For insertion at the *Bam*HI site downstream of the CAT gene, the *Bam*HI site was converted to an *Xba*I site where DNA fragments were inserted using the *Xba*I linker. A; *Apa*I, B; *Bam*HI, E; *Eco*RI, M; *Mbo*I, P; *Pst*I, St; *Stu*I, T; *Taq*I, Sc; *Sac*I, N; *Nae*I and Sal; *Sal*I.

Table I. Effect of the Length of 5′ Promoter Region of *hst* on the Transcription of CAT Gene

Plasmids	% Acetylation		
	NCC-IT	F9	NIH/3T3
pMA-CAT	1.5	0.8	0.7
pMA-CAT-ES	11.4	9.1	2.0
pPA-CAT	1.0	1.4	1.5
pPA-CAT-ES	24.1	9.0	0.9
pSA-CAT	3.8	1.8	1.3
pSA-CAT-ES	16.8	14.8	2.6

Transfection, preparation of cell lysates and CAT analysis were carried out as described in the legend to Fig. 3. Cellular proteins equivalent to 15 mU of β-galactosidase were analyzed for NCC-IT, and those equivalent to 7.5 mU for both F9 and NIH/3T3.

using a suitable probe as depicted in Fig. 1. DNase I-hypersensitive sites were detected in the 3′ region (A and B in the right panel of Fig. 2); these sites were approximately 3.5 and 4.5 kb downstream of the translational initiation site of the *hst* mRNA. No DNase I-hypersensitive site was found in the 5′ region (Fig. 2). An almost identical DNase I hypersensitivity pattern was found in genomic DNA of A431 epidermoid carcinoma cell line, which does not express *hst* (data not shown). Therefore, these DNase I-hypersensitive sites may reflect the open chromatin structure of portions of the 3′ region of the *hst*, common to many human cells, irrespective of the expression of the *hst* mRNA.

To search for regulatory regions of *hst* allowing its expression in embryonal carcinoma cell lines, we con-

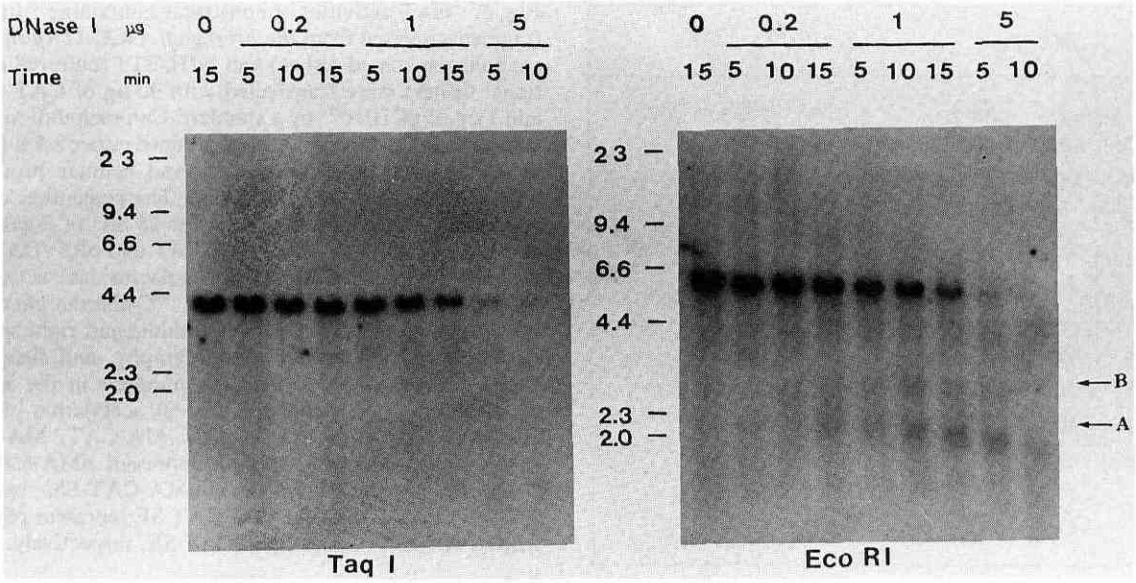

Fig. 2. DNase I hypersensitivity of the *hst* region. NCC-IT cells (3×10^7) were suspended in 5 ml of RSB (10 m*M* NaCl, 10 m*M* Tris-HCl, 3 m*M* $MgCl_2$) and incubated for 1 h on ice, then 200 μl of 5% Triton X-100 was added and the mixture was homogenized with 10 strokes in a loosely fitted Teflon homogenizer. Then 15 ml of cold RSB was added and the whole was centrifuged at 1,000 rpm for 10 min at 4°C. The precipitate was washed with cold RSB, and finally resuspended in a digestion buffer consisting of 60 m*M* KCl, 15 m*M* NaCl, 15 m*M* Tris-HCl (pH 7.4), 0.5 m*M* dithiothreitol, 0.25 *M* sucrose, 3 m*M* $MgCl_2$ and 0.05 m*M* $CaCl_2$. The nuclei suspension was diluted to $1.3\times10^7/100$ μl. DNase I digestion was carried out on 1.3×10^6 nuclei in 200 μl of digestion buffer containing 0.2 to 5 μg DNase I at 37°C for 5 to 15 min. The reaction was stopped by adding 15 μl of 10% SDS and 8 μl of 0.5 *M* EDTA. After purification and endonuclease digestion of DNA (*Taq*I for 5′ region and *Eco*RI for 3′ region), Southern hybridization was carried out with a nick-translated *Eco*RI/*Taq*I probe (see Fig. 1). The numbers on the left-hand side show the positions of size markers in kbp. A and B indicate DNase I-hypersensitive sites.

structed plasmids by inserting various candidate fragments of the *hst* region at the *Hin*dIII and/or the *Bam*HI sites of pSVOCAT (Fig. 1). At first, a 1.4 kb *Mbo*I/*Apa*I fragment (MA), which spans from −1,294 to +105 relative to the translational initiation site was used as the *hst* promoter. The resultant construct, PMA-CAT, did not show any CAT activity in either NCC-IT or F9 (Table I). Insertion of longer 5′ fragments (pEA-CAT and pBA-CAT, see Fig. 1) was also ineffective (data not shown). Other constructs containing either the first or the second intron (NS or SS) upstream of MA fragments in pMA-CAT (pNS-MA-CAT and pSS-MA-CAT) also did not show any CAT activity (data not shown).

In contrast, pMA-CAT-EE which was constructed by insertion of a 5.7 kb *Eco*RI/*Eco*RI fragment, which covers the entire third exon (EE), downstream of the CAT gene in pMA-CAT showed weak but definite CAT activity when transfected into NCC-IT (Fig. 3). This pMA-CAT-EE was inactive in NIH/3T3 where endogenous *hst* gene is transcriptionally silent. We next inserted either a 2.2 kb *Eco*RI/*Sal*I fragment (ES) or a 3.4 kb *Sal*I/*Eco*RI fragment (SE) downstream of the CAT gene of pMA-CAT (pMA-CAT-ES or pMA-CAT-SE). pMA-CAT-ES, but not pMA-CAT-SE was found to be CAT-positive. These results suggested that the DNase I-hypersensitive site A may be involved in enhancer activity on the *hst* promoter in pMA-CAT, since site A was assigned to ES fragment (see Figs. 1 and 2). The ES fragment also enhanced the promoter activity of pSVOCAT (Fig. 3). This finding suggested that the putative enhancer element(s) residing in this region was also active on heterologous promoters, since pSVOCAT has a promoter(s) active in mammalian cells.[21] The promoter activity of pSVOCAT was generally higher in NIH/3T3 than in NCC-IT. However, this enhancer showed no influence on pSVOCAT in NIH/3T3.

Next, we tested similar CAT constructs containing shorter *hst* promoter regions. pPA-CAT-ES, containing a −1,003 to +105 sequence, and pSA-CAT-ES, containing a −689 to +105 sequence upstream of the CAT gene, also showed similar CAT activity in NCC-IT and F9 (Table I). Thus, the *hst* promoter which is active in embryonal carcinoma cells should reside between −689 and the translational initiation site.

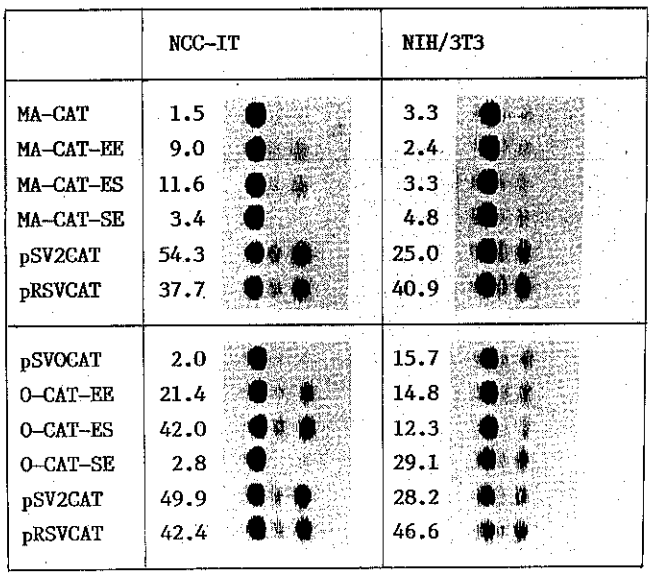

Fig. 3. CAT activities of constructs containing various DNA fragments derived from the *hst* region. NCC-IT (cultured in 10 cm collagen-coated dishes) and NIH/3T3 (cultured in conventional dishes) were transfected with 30 μg of CAT constructs and 1 μg of pCH110[22] by a standard Ca-precipitation method.[2] Exposure to Ca-precipitates was stopped either 3.5 h (NCC-IT) or 24 h (NIH/3T3) thereafter, and cellular proteins were isolated 48 h after the transfection. The percentage of acetylation in each sample is compared for 15 mU of β-galactosidase activity. For CAT assays of pSV2CAT and pRSVCAT, cellular proteins containing 750 μU of β-galactosidase activities were used. After acetylation reaction, ^{14}C-chloramphenicol (left spots) and its acetyl derivatives (middle and right spots) were separated by thin layer chromatography, and then analyzed with a β-scanner (AMBIS). The numbers in the left side of chromatograms represent the percent acetylation of chloramphenicol included in the reaction. MA-CAT, MA-CAT-EE, MA-CAT-ES and MA-CAT-SE represent pMA-CAT, pMA-CAT-EE, pMA-CAT-ES and pMA-CAT-ES, respectively. O-CAT-EE, O-CAT-ES and O-CAT-SE represent pSVO-CAT-EE, pSVOCAT-ES and pSVOCAT-SE, respectively.

Recently, Curatola and Basilico[18] reported the enhancer activity of a DNA fragment corresponding to +3,170/+3,880 of the *hst* gene (based on the numbering system used in this report) using CAT assay. This fragment was active only in embryonal carcinoma cell lines and exerted its effect on heterologous promoters.[18] The data presented here are compatible with their results.

In conclusion, *hst* promoter is inactive by itself in embryonal carcinoma cell lines as well as in NIH/3T3, and an enhancer element(s) present in the third exon is required for transcription in embryonal carcinoma cells. The chromatin structure of the region containing the enhancer may be relaxed, as suggested by the DNase I hypersensitivity. Therefore, the presence (in embryonal carcinoma cells) or absence (in differentiated cells) of a putative regulatory factor(s) should control the expression of the *hst*.

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