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Transcriptional Regulation of the Salivary Histatin Gene: Finding of a Strong Positive Regulatory Element and Its Binding Protein†

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Histatins are salivary proteins found and expressed in human salivary glands. They play a role in the non-immune system of antimicrobial defense, for instance, against *Candida albicans*. The transcriptional regulatory sequences of the histatin gene, *HIS1*, have remained obscure for a long time. Here, we cloned the putative promoter from human genomic DNA and tested it in a luciferase reporter system. This promoter is much more active in salivary gland cells than in other cell types. Analysis of deletion mutants revealed that the region encompassing −2254 to −1748 is a strong positive transcriptional element, and its functional core sequence (termed HTN27 box) works in correct and reverse orientations in synergy with downstream sequences, the region spanning −680 to +28 and a proximal promoter. The *plus* single-stranded HTN27 box is specifically bound by a 100 kDa protein that is present in HSG cells, but not in HeLa cells. These findings indicate that the regulation of the histatin gene expression may be intricate, and it seems to have a cell-type preference in the salivary gland cells.

Key words: DNA-binding proteins, histatin, positive regulatory element, saliva, transcriptional regulation.

Abbreviations: DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; HSG, human salivary gland; MEME, minimum essential medium Eagle.

INTRODUCTION

Saliva is associated with oral non-immune defense system. Some salivary proteins have antibacterial and antifungal activities (*1*). Histatins are histidine-rich, cationic proteins of about 3–4 kDa; they are secreted by salivary (submandibular, sublingual and parotid) glands in humans and higher primates (*2*, *3*) and are generally present in human oral tissues (*4*). There are 12 proteins in the histatin family and they are present in healthy adult saliva at concentrations of 50–425 μg/ml (*5*). Histatins 1 and 3 (38 and 32 amino acids, respectively) are full-length proteins encoded by the two closely related loci *HIS1* and *HIS2* on human chromosome 4, respectively (*6*). Histatin 1, a phosphoprotein, was found as adsorbing to hydroxyl apatite and enamel powders and inhibited the precipitation of calcium phosphate salts in saliva (*7*–*9*). Histatin 5 (24 amino acids) is probably a derivative of histatin 3, either a proteolytic product thereof or a product of a post-transcriptional modification of histatin 3 mRNA (*6*). The other members of the family, histatin 2 and histatins 4–12, are found in parotid saliva and are generated by proteolytic processing during secretion (*10*, *11*).

Histatins 1 and 3 have antimicrobial activity against *Candida albicans* at their physiological concentrations of 15–50 μM (*3*, *9*, *12*–*14*). The fungicidal mechanism of histatin 5 is a multistep process. First, histatin 5 binds to a cell-envelope protein of *C. albicans* [identified as the 70 kDa heat shock protein (Ssa1/2p)] and a 67–70 kDa protein of *Saccharomyces cerevisiae* (*15*, *16*). Second, histatin 5 is internalized into *C. albicans* and targeted to the energized mitochondrion (*17*), where it induces high levels of reactive oxygen species in the yeast cells, reduction of the total cell volume and cell-cycle arrest (*18*, *19*). Histatin 5 has also been reported to cause non-lytic release of ATP from *C. albicans* leading to cell death (*20*) and to inhibit both a trypsin-like protease produced by *Bacteroides gingivalis* and a cysteine protease clostripain produced by *Clostridium histolyticum* (both of which are oral bacteria suspected of causing periodontal disease) (*21*, *22*). Furthermore, histatins induce the release of histamine from rat mast cells and have an effect on the growth of rabbit chondrocyte (*23*–*25*). A histatin-resistant *C. albicans* strain is five times less susceptible of killing by histatin 3 at physiological concentrations than the wild-type strain, and this is not due to alterations in the binding, internalization, or degradation of histatin 3 or in the efflux of ATP (*26*). In the light of these reports, we conclude that histatins

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have a very important physiological function of defense in the oral cavity.

Cell type, tissue-specific and/or preferential gene expressions are proving very important and complicated control systems of development and morphogenesis. Classical examples of such controlled expression, and of their inherent physiological importance, are the expression of insulin, immunoglobulin, chymotrypsin, actin, ATPaseII, KSP-cadherin and testisin (*27–33*). Control of expression may take several forms: time, location and strength. Usually, such control systems involve regulatory DNA sequences (for instance, promoters) and often their cellular binding factors, which may be specific to certain cells or tissues or, in turn, may be expressed in a temporal manner.

So far, the cDNAs of human histatins have been isolated, and their expressions have been observed in salivary glands (*34*). However, the histatin gene and, in particular, its promoter region have not yet been cloned and studied. In this study, we have cloned the upstream region of the *HIS1* (also known as *HTN1*) gene and analysed its transcriptional regulation elements. We found a positive regulatory element within 2.3 kb upstream from the first exon, which element stimulates transcription strongly in HSG salivary gland cells, but not in other cell types.

MATERIALS AND METHODS

Cell Culture—HeLa (human cervical carcinoma), HEK293 (human kidney), SCCTM (human oral epithelial carcinoma), COS-7 (monkey kidney) and NIH3T3 (mouse fibroblasts) cells were maintained in Dulbecco's modified Eagle medium (DMEM, Sigma) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin G and 100 μg/ml streptomycin. HSG (human salivary gland) and THP-1 (human monocyte) cells were maintained in minimum essential medium Eagle (MEME) alpha modification (Sigma) and in RPMI1640 (Sigma) containing 10% FBS, 0.292 mg/ml L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin, respectively. Cells were cultured at 37°C in 5% CO_2 and 95% humidified incubator.

Oligonucleotides—All primers for PCR were designed on the basis of the sequences from 'accession number AADB01027612' of the National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov/). The oligonucleotides used in this study are listed below.

hHstproA: 5′-GCTCGAGGTCAGTGAAGCTGAAGTGAAGTCTCAAGAGATG-3′

hHst-(−2254)F: 5′-GCTGCAGGGTTTGATTGTAGTTTTTAAATGAG-3′

hHst-2-1: 5′-GGGAATTCGGAAGAAATGAGAGGAGGCAG-3′

hHst-2-A: 5′-GGGGTACCAACTACAGGGCTCAAGCAAACC-3′

hHst-2-2: 5′-GGGAATTCTTAGGCACGAGGACCACCTG-3′

HTN27WT(+): 5′-AATTCCCTAATATTGTTAGAAAAACGAACTAGG-3′

HTN27WT(−): 5′-AATTCCTAGTTCGTTTTTCTAACAATATTAGGG-3′

HTN27M(+): 5′-AATTCTTCTCCCCTGTTAGAAAAACGAACCCTG-3′

HTN27M(−): 5′-AATTCAGGGTTCGTTTTTCTAACAGGGGAGAAG-3′

hHst-2(−2086)R: 5′-GGAATTCCTAGTTCGTTTTTCTAACAATATTAGG-3′

hHst-1: 5′-GGAATTCGATTCACATGAAAAGAGACATCATGGGTATAG-3′

hHst-A: 5′-GGATATCAATTGTCATATAGATAATTTGATCCA TAGTCCC-3′

hHst-RT-1: 5′-CGAGAATTTCCATTTTATGG-3′

hHst-RT-A: 5′-ATTACTCAGAAACAGCAGTG-3′

H1: 5′-GGTTTGATTGTAGTTTTTAAATGAGTATATTGACATCTGC-3′

H2: 5′-ATGAGTATATTGACATCTGCCATATCTTACCTGTTAGTTT-3′

H3: 5′-CATATCTTACCTGTTAGTTTGCTGTTTGGTAGTGTGCTT T-3′

H4: 5′-GCTGTTTGGTAGTGTGCTTTTGGACTATTAGTTTGCTTTT-3′

H5: 5′-TGGACTATTAGTTTGCTTTTTGTGCACTAGAACATGAATA TCTTA-3′

H6: 5′-ACTAGAACATGAATATCTTAGATCCACTGGAACTGTACCT-3′

H7: 5′-GATCCACTGGAACTGTACCTAATATTGTTAGAAAAACGAAC TAG-3′

H9: 5′-TGACATCTG CCATATCTTAC-3′

H12: 5′-CCTAATATTGTTAGAAAAACGAACTAG-3′

C1: 5′-CCGGGCTTGATGACCTCAGGAATGTCAAGAAGCTAGGTGG-3′

C2: 5′-CCGGG CTTGATGAC CTCAGG-3′

Sp1: 5′-ATTCGAT CGGGGCGGGGCG AGC-3′

Cloning of Human Histatin Promoter and Plasmid Constructions—A fragment of the histatin upstream sequence (2282 bp) was amplified by PCR from 50 ng of human genome DNA from lingual mucosal cells (*35*) with 1.75 U of Expand High Fidelity polymerase (Roche) using 100 pmol each of the forward primer hHst-2(-2254)F and of the reverse primer hHstproA. The fragment ligated to a *Kpn* I linker (Takara) was digested with *Kpn* I and *Xho* I followed by insertion into pUC-Luc (pHst-LucWT, Fig. 1B). Ten independent plasmids were cloned and sequenced (all resulted identical). The sequence was submitted to DDBJ (accession number AB430585). The deletion mutants of the histatin promoter-luciferase plasmids were obtained as follows. Ten micrograms of pHst-LucWT were digested with *Eco*R I and reacted with Exonuclease III (Takara, 90 U), Mung Bean nuclease (Takara, 25 U) and Klenow fragment (Takara, 1 U). The DNA was ligated to a *Kpn* I linker and digested with *Kpn* I and *Xho* I. The mutant DNAs were ligated into *Kpn* I and *Xho* I sites of pUC-Luc (pHst-LucΔ1–3: shown as 'Δ1'–'Δ3' in Fig. 2A, respectively). Plasmids pHst-LucΔ4 and pHst-LucΔ5 ('Δ4' and 'Δ5' in Fig. 2C, respectively) were obtained as follows. Using pHst-LucWT as a template, we amplified by PCR a fragment of 338 bp with primers hHst-2-1 and hHst-2-A, and a fragment of 169 bp with primers hHst-2-2 and hHst-2-A. These two fragments were inserted into the *Eco*R I and *Kpn* I sites of pHst-LucΔ1, respectively. Plasmid pHstpro-Luc ('pro' in Fig. 6C): after changing the *Nde* I site to *Kpn* I in pHst-LucWT, the *Kpn* I-*Bam*H I fragment was isolated

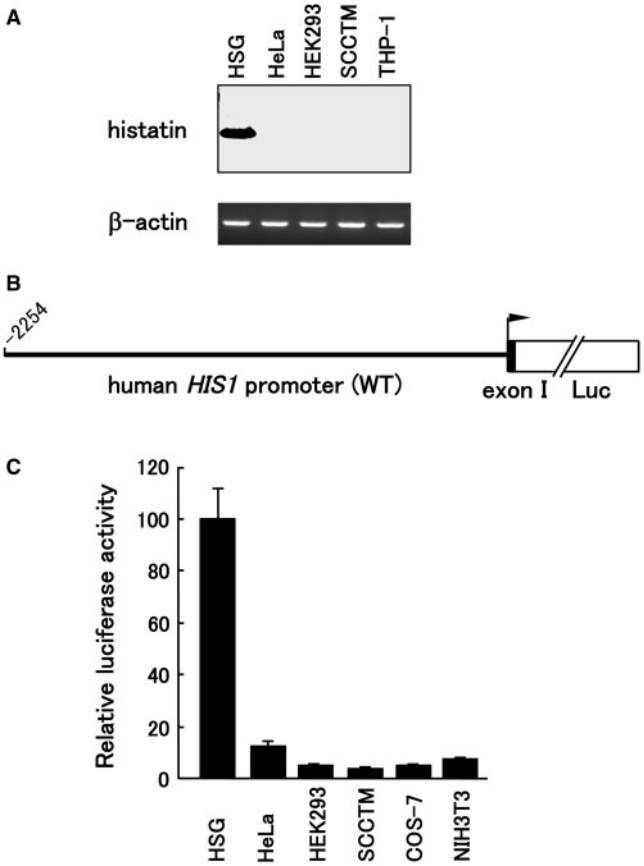

Fig. 1. **Expression profile of the histatin gene and schematic representation of the reporter construct and transcriptional regulation of the promoter.** (A) RT-PCR was carried out using the purified RNAs from human cell lines, HSG, HeLa, HEK293, SCCTM and THP-1. For histatin, PCR samples were electrophoresed on 1% agarose gel followed by southern-blotting analysis. For β-actin, the PCR samples were electrophoresed on 0.8% agarose gel followed by staining with ethidium bromide. (B) The sequence of the histatin gene covering the fragment spanning positions −2254 and +28 was ligated to a luciferase gene. (C) The reporter construct and a standard plasmid (pRSV-β-gal) were co-transfected into various cells. The detail procedures were described in 'Materials and Methods' section. The transcriptional activities indicate as percentage values of those of WT. Bars represent the means with SDs of duplicated samples. Shown are representatives of three identical series of experiments with essentially identical results.

and cloned into pUC18. Plasmids pHst-LucΔ4WT(+), (−), pHst-LucΔ4M(+), (−), pHstpro-LucWT(+), (−) and pHstpro-LucM(+), (−) (i.e., 'Δ4WT(+), (−)', 'Δ4M(+), (−)', 'proWT(+), (−)' and 'proM(+), (−)' in Figs 5B and 6C, respectively) were obtained as follows. Each of the following oligonucleotide pairs was annealed and inserted into the *Eco*R I site of pHst-LucΔ4 and pHstpro-Luc, respectively: HTN27WT(+) and HTN27WT(−) for HTN27 box wild type and HTN27M(+) and HTN27M(−) for HTN27 box mutant. The orientation of the inserted oligonucleotides was determined by sequencing. For the construction of pCR-BluntII-Hstpro(169), the promoter spanning −2254 to −2086 (169 bp) was amplified by PCR from pHst–LucWT with primers hHst-(−2254)F and hHst-2(−2086)R. The fragment was inserted into pCR-BluntII TOPO (Invitrogen). For the construction of pCR-BluntII-*HIS1*, a fragment of *HIS1* cDNA was amplified by PCR with primers hHst-1 and hHst-A and a human submaxillary cDNA library (Clontech) as a template. The amplified DNA was ligated to pCR-BluntII-TOPO.

RT-PCR and Southern Blotting—Purification of total RNA from the cells (5×10^6) and RT-PCR were carried out as described in Imamura *et al*. (*36*), using the specific primers hHst-RT-1 and hHst-RT-A. The samples were separated by electrophoresis in a 1% agarose gel, and the DNAs were transferred to a nylon membrane. An *Eco*R I fragment of the *HIS1* cDNA was isolated from pCR-BluntII-*HIS1* and labelled by Gene Images AlkPhos Direct Labelling and Detection System (GE Healthcare). The hybridization of the resulting probe to the samples on the nylon membrane and following procedures were carried out in accordance with the instructions of the manufacturer of above system. As a control of nucleic acid quantity, amplified samples for β-actin were electrophoresed on 0.8% agarose gel and stained with ethidium bromide.

Transfection and Luciferase Assay—One microgram of each of the luciferase plasmids and 0.1 μg pRSV-β-gal (standard plasmid) were individually mixed with TransIT-LT1 reagents (Mirus). The mixtures were transfected to 3×10^5 cells. Two days after transfection, the cells were harvested and lysed. Luciferase and β-galactosidase activities in the lysates were measured as described previously (*36*), and the luciferase activity in the various samples was compared after normalization on the basis of the standard (β-galactosidase activity).

Gel Mobility Shift Assay—Nuclear extracts from HSG and HeLa cells were obtained as described previously (*37*). The *EcoR* I-*Pst* I fragment (169 bp) of the promoter (−2254 to −2086) was isolated from pCR-BluntII-Hstpro(169). The DNA was end-labelled with Biotin 3′ End DNA Labeling kit (Pierce). One microgram of nuclear extracts and 20 fmol of probe were mixed and treated with Lightshift Chemiluminescent EMSA kit (Pierce). After electrophoresis in a 4% native polyacrylamide gel (29:1 cross-linking), the DNAs were transferred to a nylon membrane. The filter was irradiated with UV light with a FUNA-UV-LINKER FS-1500 apparatus (Funakoshi, optimal crosslink: 120 mJ/cm^2). The DNA bands on the filter were detected with streptavidin-HRP. Gel mobility shift competition assays were carried out by adding non-labelled probes as competitors in 20-, 100-, or 200-fold molar excess. The competitor oligonucleotides covered the following segments of the promoter region: H1 (−2254 to −2215), H2 (−2234 to −2195), H3 (−2214 to 2175), H4 (−2194 to −2155), H5 (−2174 to −2130), H6 (−2149 to −2110), H7 (−2129 to −2086), H9 (−2224 to −2205), H12 (−2112 to −2086, called HTN27 box), controls C1 and C2 (non-specific), and Sp1. These oligonucleotides were annealed with their cognate antisense ones and used. Further oligonucleotides used as competitors for H12 are described in Fig. 4A.

UV-Crosslinking Assay—The proteins were allowed to bind to the DNAs in the same way as described for the gel mobility shift assay. The samples were irradiated

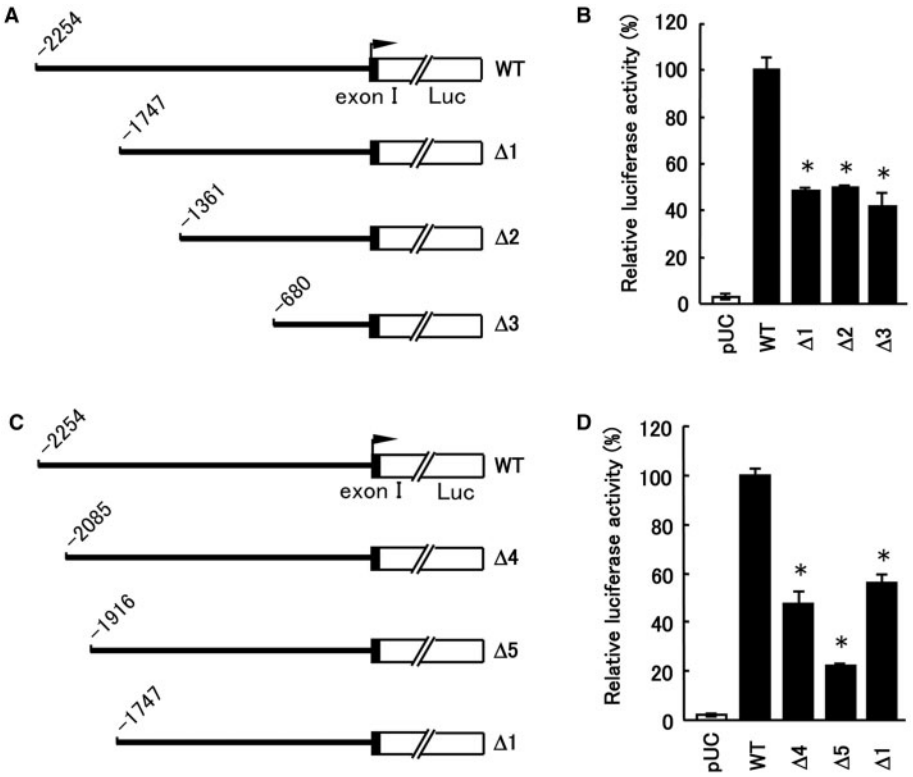

Fig. 2. **Construction of deletion mutants and their transcriptional regulation.** (A) The promoter DNA was digested with Exonuclease III from the −2254 position. These resulting deletion mutant fragments were ligated to the luciferase gene and sequenced. The deletions provided construct with progressively shorter sequences of the promoter region, now starting at positions −1747, −1361 and −680, respectively. (B) Each of the deletion mutants reporter construct was tested in HSG cells. The transcriptional activities are indicated as % values in relation to those of WT in HSG cells (set as 100%). (C) The deletion fragments of −2085 to −1748 and −1916 to −1748 were amplified by PCR and ligated at position −1747 of Δ1, yielding Δ4 and Δ5, respectively. (D) The constructed reporter plasmids were tested as described for in Materials and Methods. $^{*}P < 0.01$ compared with the activity of WT, respectively (Student's *t*-test).

at 254 nm of UV for 40 min on ice followed by the addition of a sample buffer for SDS–PAGE. The samples were electrophoresed on 10% SDS-polyacrylamide gel and transferred to a nylon membrane, which were then treated in the same way as described for the 'Gel mobility shift assay'. The sequences of the oligonucleotides used are indicated in Fig. 4A.

RESULTS

Expression Pattern of the Histatin Gene for Various Cell Types—We have examined the expression pattern of the gene in various cells by RT-PCR and southern blotting. As can be seen in Fig. 1A, the gene was expressed in HSG cells, but only very weakly in HeLa, HEK293, SCCTM and THP-1 cells. This was also expressed at least 20-fold higher in HSG cells treated with 5-azacytidine, but not HeLa cells (data not shown, *38*). These results seem that the histatin gene is expressed preferentially in salivary gland cells.

Cloning and Transcriptional Regulation of the Histatin Promoter—To investigate the transcriptional regulation of the histatin gene, we have cloned the −2254 to +28 region of this gene (the promoter region) and used it to construct a luciferase reporter plasmid (WT, Fig. 1B), which we tested in HSG, HeLa, HEK293, SCCTM, COS-7 and NIH3T3 cells. As shown in Fig. 1C, the transcriptional activity in HSG cells ranged from 5.5 to 16 fold higher than that in the other cells. These results suggest that this promoter regulates expression in a cell-type preferential manner.

Transcriptional Regulation of Deletion Mutants—Next, we identified the elements in this promoter responsible for transcriptional activity. Deletion mutants were produced from its 5′-region (−2254 position) and linked to the luciferase gene. As shown in Fig. 2A, the deletion mutants comprise the first 1747, 1361 and 680 upstream from the transcription initiation site, respectively. These constructs were tested in HSG and HeLa cells. All the deletion mutants displayed transcriptional activity in HSG cells (Fig. 2B), but hardly any activity in HeLa cells (data not shown). Moreover, we found that the region encompassing −2254 to −1748 works as a strong positive regulatory element of transcription (possibly by cooperating with its downstream region), because the activities of the Δ1, 2 and 3 decreased to ∼40–50% compared with that of WT ($P < 0.01$). These results indicate that this promoter contains the strong positive regulatory regions, and we infer that expression in HSG cells must be regulated intricately.

Deletion Mutants of the −2254 to −1748 Region and Their Transcriptional Activities—The strong positive regulatory element appear to cooperate with downstream elements was found between positions −2254 and −1748. Significant activation of the region −680 to +28 was observed (Fig. 2B). A further plasmid, comprising the region −2254 to −681 and −88 to +28 (a proximal promoter) linked to the luciferase gene, yielded an activity 2.4-fold higher than that of WT (data not shown). Therefore, the region −2254 to −681, which includes the region −2254 to −1748, appears to regulate the proximal promoter. We therefore decided to dissect this region more finely and created two further deletions (recessed up to positions −2086 and −1917, respectively, Fig. 2C). All the deletion mutants displayed transcriptional activities in HSG cells (Fig. 2D), but hardly any activity in HeLa cells (data not shown). In particular, the region from −2254 to −2086 had a positive effect on the downstream region it controlled: There was a statistically significant difference between the activity of WT and that of Δ1, 4 and 5, respectively ($P < 0.01$). These findings indicate that the region from −2254 to −2086 may have very important functions for strong expression of the histatin gene.

Presence of Nuclear Proteins Bound to the −2254 to −2086 Region—In order to investigate the presence of nuclear factors bound to the −2254 to −2086 region, gel mobility shift competition assays were performed with HSG and HeLa nuclear extracts. The competitors (H1 through H7) were double-stranded oligonucleotides designed to cover different portions of this region. As shown in Fig. 3A, in the samples with no addition of competitors, there are two bands in both HSG and HeLa cells (bands 'I' and 'II'). Upon addition of the competitors, the band 'I' was strongly competed out with H1, H2, H5 and H6 in both cell types, but not with a non-specific control 1 (C1). Band 'II' was strongly competed out in both cell types when the H2 competitor was added. In contrast, upon addition of the H7 competitor, band 'I' was weaker in HSG than in HeLa cells, suggesting that H7 may bind to a protein that is more present or more active in HSG than in HeLa extracts. In order to rule out an artifact due to the different concentration of the two nuclear extracts, a probe containing the binding site for Sp1 (a transcription factor expressed constitutively in virtually all cell types) was used in a control gel mobility shift competition assay (Fig. 3F). The binding behaviour of both in the presence and absence of Sp1WT competitor and of a non-specific control 2 (C2) was identical. Thus, we confirmed that the amounts of total protein in both extracts were comparable. We concluded that H7 (−2129 to −2086) contains a sequence bound by a factor found in HSG, but scarce in HeLa cells.

Presence of Nuclear Factors Bound to Double- and Single-Stranded −2224 to −2205 and −2112 to −2086 Regions—In order to further narrow the investigation, we identified two oligonucleotides, H9 (the region −2224 to −2205) and H12 (the region −2112 to −2086), that could compete (in 200 molar excess) with H2 and H7 probes for binding to HSG and HeLa nuclear extracts (data not shown). The results obtained with the H9 and H12 probes are shown in Fig. 3B and C, respectively. It is evident that both extracts display the same binding behaviour in the presence and absence of competitors. In addition, the experiments with the extracts from HEK293, SCCTM and THP-1 cells were also shown to the same results (data not shown).

It is also known that single-stranded binding proteins may interact with transcriptional regulatory elements. We found that when the *plus* stranded H9 (H9(+)) probe was used, three bands indicated by arrowheads in Fig. 3D were detected with the HSG extracts and also the THP-1 ones (data not shown). When the *minus* stranded H9 (H9(−)) probe was used, HSG, HeLa (Fig. 3D) and other cell extracts (data not shown) display the same behaviour. Moreover, when the *plus* stranded H12 probe (H12(+)) was used, the HSG extracts gave rise to binding (two bands): no or the very weakly bands were observed with HeLa (Fig. 3E) and other cell extracts (data not shown), respectively. The results with the *minus* stranded H12 (H12(−)) probe and the extracts from above all cells showed the similar pattern in Fig. 3E. The bands observed with both the H9(+) and the H12(+) probes using the HSG extracts correlate with sequence-specific binding (abolished by addition of 200-fold H9(+) and H12(+) competitors, but not a non-specific competitor). These results indicate that nuclear proteins bound to the *plus* single-stranded −2112 to −2086 region are predominantly present in HSG cells.

Essential Nucleotides in the −2112 to −2086 Region Indispensable for Binding—We then determined which of the nucleotides in the H12(+) is essential for binding the cognate proteins. Gel mobility shift competition assay was performed in the presence and absence of various competitors, which are listed in Fig. 4A. All competitors were able to cancel the binding represented by the upper band, whereas competitors M1, M2 and M7 were unable to compete for the binding represented by the lower band (Fig. 4B), indicating that the nucleotides which in these competitors are mutated vis-à-vis the wild-type H12(+) sequence are crucial for binding. We concluded that sequences −2112 to −2105 (CCTAATAT) and −2088 to −2086 (TAG), separated by a sequence of 16 nucleotides corresponding to the −2104 to −2089 region or mutants thereof, are essential for binding. (In contrast, when the H9(+) probe was used in combination with the same competitors, no sequence-specific binding proteins were observed: data not shown).

Molecular Weight of the Binding Factors—Next, we measured the molecular weight of the H12(+)-specific binding proteins by UV-crosslinking assay using the H12(+), M1, M2 and M7 probes with HSG nuclear extracts (Fig. 4C). Proteins of ~60 and 55 kDa recognized the wild-type sequence of H12(+) as well as its mutants M1, M2 and M7. However, only the wild type was bound by a protein of ~100 kDa. This result suggests that the 100 kDa protein binds to the sequences (CCTAATAT—TAG), which have been recognized as essential nucleotides.

Transcriptional Activities of HTN27 Box—We have determined how the region −2112 to −2086 (now renamed HTN27 box, Fig. 5A) regulates the transcriptional activity of the region −2085 to +28. We constructed reporter plasmids containing the wild type and

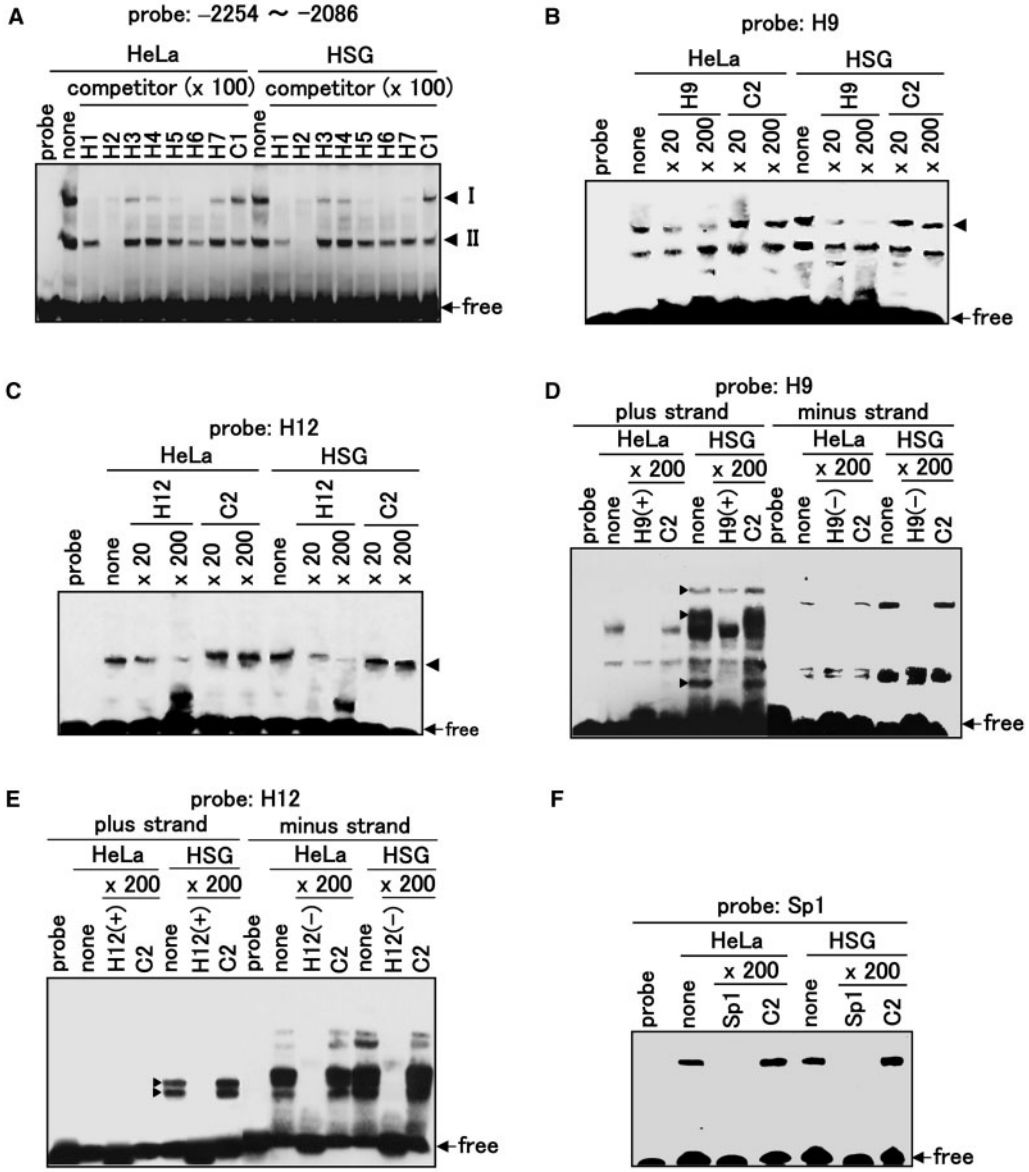

Fig. 3. **Gel mobility shift competition assay for the positive regulatory element of the transcription.** (A) Nuclear extracts from HeLa and HSG cells were mixed with a biotin end-labelled probe of the −2254 to −2086 region of the promoter, in the presence and absence of 100-fold excess unlabeled competitors against the probe. The competitors were oligonucleotides corresponding to different (overlapping) portions of the probe. C1 was an unrelated sequence used as a negative competitor control. The detail procedures were described in Materials and Methods. The most significant sequence-specific bands are indicated by arrowheads of I and II. (B and C) Gel mobility shift competition assays were carried out using H9 and H12 probes, respectively. The arrowheads indicate sequence-specific bands. (D and E) Gel mobility shift competition assays with single-stranded H9 and H12 probes. The arrowheads indicate sequence-specific bands in HSG cells. (F) Gel mobility shift competition assay with a Sp1 probes to confirm that both the nuclear extracts from HeLa and HSG cells used contained comparable levels of protein for the above experiments. C2 was an unrelated sequence used as a negative competitor control.

mutant HTN27 sequences incorporating the mutations of M1, M2 and M7 (in forward and reverse orientations, Fig. 5B) and tested them in HSG and HeLa cells. Figure 5C shows that the correct [Δ4WT(+)] and reverse [Δ4WT(−)] orientations of the wild-type HTN27 box stimulated the transcriptional activity to ~90% and 70% compared with WT in HSG cells, respectively. Even in the reverse orientation, the activity of the wild-type HTN27 box was statistically significant ($P < 0.01$). However, none of the constructs had any significant transcriptional activity in HeLa cells (data not shown). In sharp contrast, addition of the mutant HTN27 box in either orientation [Δ4M(+) and Δ4M(−)], not only did not raise the transcriptional activity of Δ4, it actually dramatically inhibited it. From these results, we infer that the presence of the HTN27 box with the wild-type sequence and in the correct orientation against the region −2085 to +28 contribute to the activity of strong transcription in HSG cells.

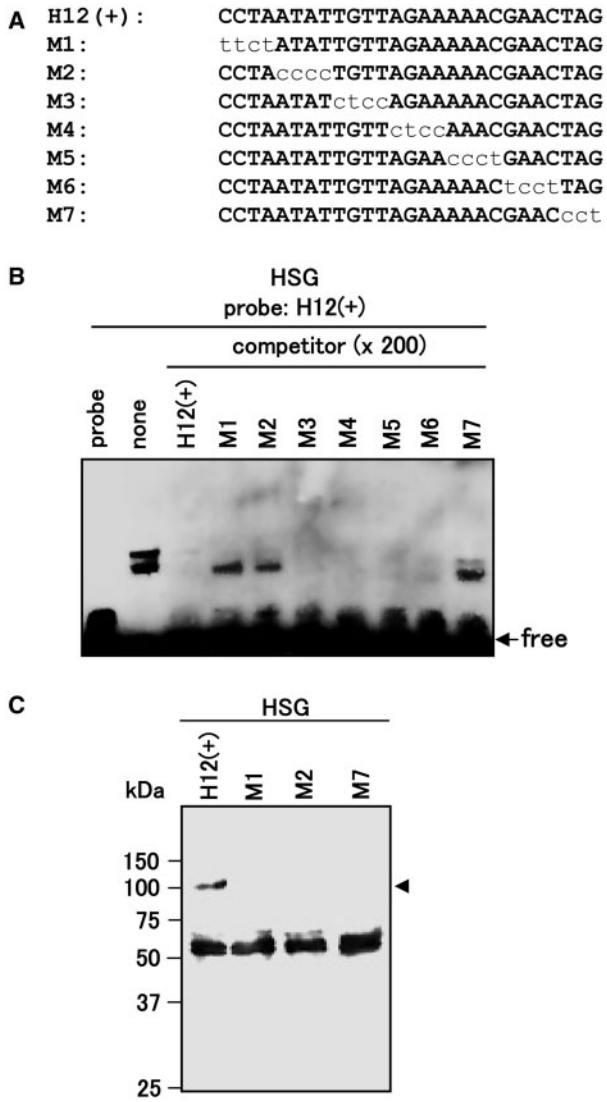

Fig. 4. **Sequence-specific-binding proteins for the single-stranded DNA of the promoter.** (A) The sequences of the wild type and mutant *plus* single-stranded H12 [H12(+)] are shown. Bold and capital letters indicate the wild-type sequences, while small letters indicate the mutant residues. The mutant sequences have been called M1 ~ 7 for H12. (B) Gel mobility shift competition assays with end-labelled H12(+) probes, using nuclear extracts from HSG cells, in the presence and absence of 200-fold excess competitors chosen from the sequences listed in Fig. 4A. (C) Proteins bound to the H12(+) were detected by UV cross-linking assay. Single-stranded, end-labelled probes of wild-type H12(+) and of its mutants M1, 3 and 7 were mixed with the nuclear extracts from HSG cells, respectively, and the following procedures were described in Materials and Methods. The numbers at the left of the figure indicate the molecular weights of protein size markers.

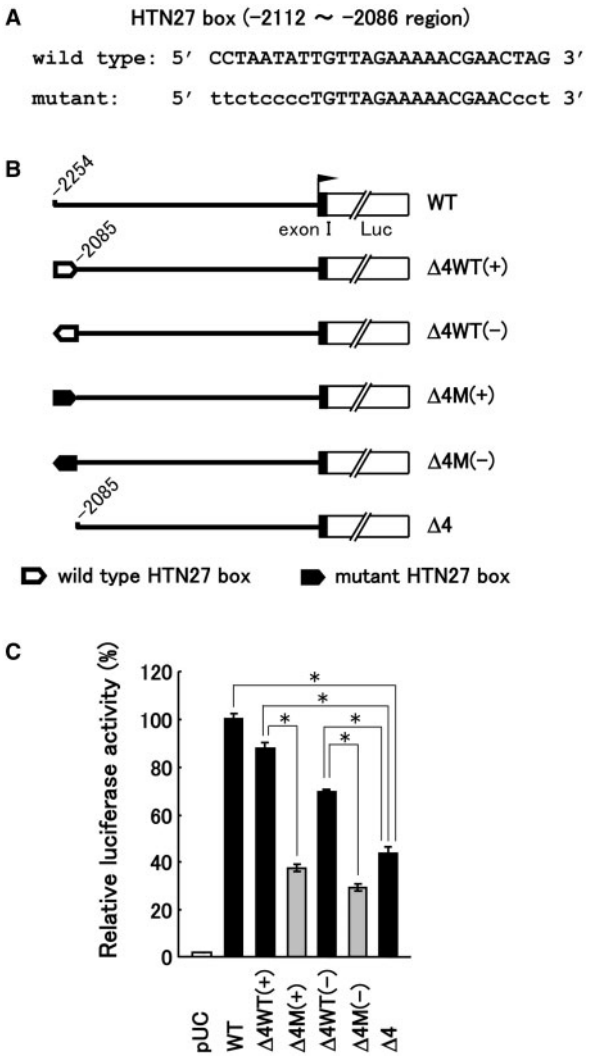

Fig. 5. **Effect of the HTN27 box on the transcriptional activity of the −2085 to +28 region.** (A) The sequences of a wild type and a mutant of the −2112 to −2086 region (named HTN27 box) are shown. The mutant incorporates all the modifications that result in the loss of the protein-binding activity. Bold and capital letters indicate the wild-type sequences, while the small letter indicates the mutations. (B) The wild type and mutant HTN27 box were linked to the −2112 to +28 region and constructed reporter plasmids in the correct and reverse orientations. (C) The plasmids obtained in (B) were tested in HSG cells by luciferase assay. The transcriptional activities were indicated as percentage values of those of WT in HSG cells (set as 100%). $^{*}P < 0.01$ compared between the activities indicated, respectively (Student's *t*-test).

We next analysed how the HTN27 box regulates the transcriptional activity of the region (−680 to +28) and of the proximal promoter. On its own, the region −680 to +28 has some basic activity (since it contains the promoter), which amount to about 50% of the activity of the whole −2254 to +28 region (Fig. 2B). As shown in Fig. 6, in HSG cells the addition to the −680 to +28 region of a wild-type HTN27 box, but not a mutant one, in both orientations activated the transcription in HSG cells. The differences among the activities of Δ3WT(+), (−) as compared to Δ3, and of proWT(+), (−) as compared to pro, were statistically significant ($P < 0.01$). In contrast, only marginal activity was observed in HeLa cells (data not shown). These results confirm that the HTN27 box works as a positive transcriptional regulatory element in HSG cells.

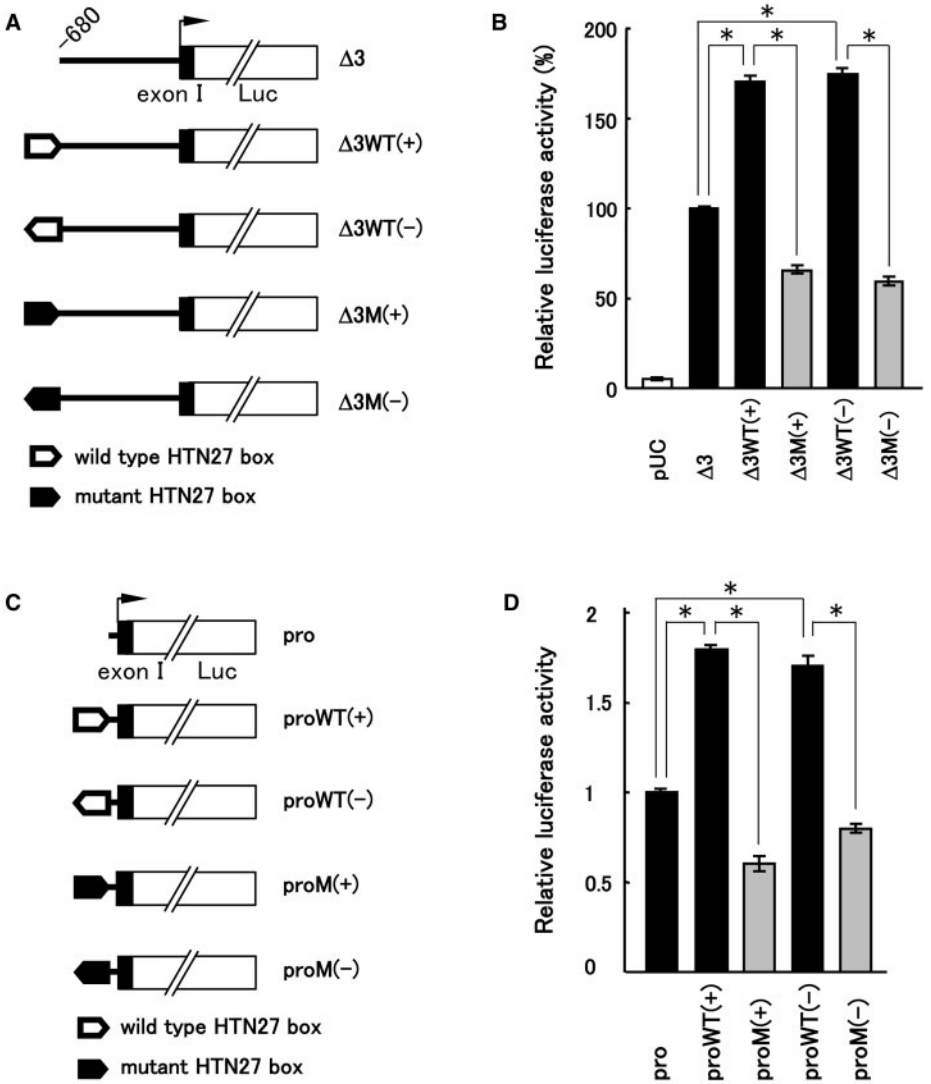

Fig. 6. **Effect of the HTN27 box on the transcriptional activity of the −680 to +28 and the proximal promoter region.** (A and C) The wild type and mutant HTN27 box were linked to the −680 to +28 region and the proximal promoter and constructed reporter plasmids in the correct and reverse orientations. (B and D) The plasmids obtained in (A) and (C) were tested in HSG cells by luciferase assay. The transcriptional activities were indicated as percenatge and fold values of those of Δ3 (B) and pro (D) in HSG cells, respectively. $^{*}P < 0.01$ compared between the activities indicated, respectively (Student's *t*-test).

DISCUSSION

The transcriptional regulation of the salivary histatin gene has not yet been clarified. In this study, we have determined that the *HIS1* promoter region (−2254 to +28 region) drives transcription strongly in human salivary glands cells and only marginally in other cell types (Fig. 1), which correlates with the observation that histatins are secreted primarily in the saliva (*2*–*4*). The analysis of the deletion mutants of the promoter region (Fig. 2) revealed the existence of the strong positive regulatory element, with a stimulating (enhancing) element spanning positions −2254 and −2086 cooperating with its downstream. Here, we have focused on the stimulating element, and in particular, on its effect on the core sequence that regulates the transcription.

Immunogold-silver staining experiments revealed the localization of histatin proteins in intercalated duct cells, although a large amount of those existed in acinar cells (*39*). HSG cells, which are derived from submandibular intercalated duct cells, differentiate into acinar(-like) cells by the culture conditions (*40*, *41*). We have confirmed that the level of histatin mRNA was markedly increased when HSG cells were cultured with 5-azacytidine (data not shown, *38*), suggesting that a low-level expression of histatin in HSG cells was observed under non-stimulating (our culture) conditions [like as a salivary protein cystatin (*41*)]. Therefore, we had used the cells in this study.

Within the stimulating element (−2254 to −2086), we have identified two sequences, H9 (−2224 to −2205) and H12 (HTN27 box: −2112 to −2086), that interact with

proteins present in both HSG and HeLa cells. Many factors appear to bind the double-stranded and the *minus* single-stranded probes of these two sequences and are unlikely to account for the strong transcriptional activity of these regions in HSG cells. In sharp contrast, there exist some proteins that bind the *plus* single-stranded probes of either sequences specifically and are found in HSG cells, but not HeLa cells (Fig. 3). Of these proteins, we observed that those who bind the H9(+) sequence are not affected by mutations of the binding sequence (data not shown), nor are those that bind to the H12(+) (upper band) (Fig. 4B). This is odd, because binding was found (Fig. 3D and E) to be sequence specific (in relation to unrelated competitors). This probably means that these factors recognize the overall structure of the region, but not any fine difference in the sequence. Therefore, it is quite unlikely that they play a significant role in transcriptional regulation according to classical canons. However, a 100 kDa factor that binds to the H12(+) sequence (lower band, Fig. 4C) is sensitive to mutations. Interestingly, it is present at the extremities of the sequence [sequences −2112 to −2105 (CCTAATAT) and −2088 to −2086 (TAG)], whereas the central sequence of 16 nucleotides corresponding to the −2104 to −2089 region seems to be able to accommodate quite extensive mutations.

Previous studies have reported that single-stranded DNA binding proteins were identified and characterized as positive and negative transcriptional regulators, and participated in cell-type specific controls (*37*, *42–45*). Moreover, nucleolin, which is a 100 kDa protein, specifically binds to the single-stranded nucleotides and the base-unpairing region of at least certain MARs and binds the sequence 5′-TCTTTAATTT*CTAATATATTTAG*A-3′, which closely recall the extremities of the HTN27 box (CCTAATAT and TAG), and in which the sequence AATATAT has been described as the core unwinding sequence (*46*). By super-shift assay using anti-nucleolin antibody and nuclear extracts from HSG cells, we were able to rule out that 'our' 100 kDa protein is one and the same as nucleolin (data not shown).

Initiation of transcription involves complex interactions between nuclear proteins and *cis*-acting elements located in the promoter and enhancer regions (*47*). The presence of HTN27 box in the correct and reverse orientations of the upstream from positions −2086, −680, or the proximal promoter ensures the transcriptional activity in HSG cells (but not in HeLa cells). There may be cross-talk between the proteins (including the 100 kDa) bound to the HTN27 box and those bound to the proximal promoter. We do not yet know the identity of the 100 kDa: we are about to clone its coding sequence in order to identify it and characterize its function.

It has been reported that the histatin levels tend to be lower in AIDS patients than in healthy adult controls (*5*), which may account for the defective antifungal activity in AIDS patients. The present findings provide new clues for our understanding of the mechanisms of transcriptional regulation and expression followed by proteins synthesis and secretion of salivary protein histatins. The findings may provide the basis for developments towards the prevention of infections in the oral cavity.

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CONFLICT OF INTEREST

None declared.

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