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Identification of a pH-responsive DNA region upstream of the transcription start site of human *NBCe1-B*

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Abstract

In rodent incisors two distinct stages of enamel formation can be identified visually based on cell morphology: the secretory stage and the maturation stage. The expression profiles of many genes characterize both stages, including the bicarbonate transport protein NBCe1. Bicarbonate is a requirement for the mineralizing enamel matrix to buffer excessive protons that form as a consequence of hydroxyapatite formation. *NBCe1-B* mRNA is up-regulated during the maturation stage of amelogenesis, where hydroxyapatite formation predominates. In this study, a presumed 572-bp *NBCe1-B* promoter region was subcloned into a reporter construct, and within this 572-bp region of DNA we characterized a 285-bp segment that shows an increase of ~ 2.3-fold in gene-transcription activity when transfected into ameloblast-like cells and cultured in medium maintained at pH 6.8 (vs. pH 7.4). A presumed pH-responsive transcriptional factor-binding domain(s) thus resides in the 285-bp *NBCe1-B* promoter region where candidate domains include the nuclear factor of kappa light polypeptide gene enhancer in B-cells1 (NFkB1), jun proto-oncogene (JUN), and tumor protein p53 (TP53)-binding sites. Mutagenesis studies identify that both the NFkB1- and TP53-binding sites are responsive to changes in the extracellular pH. These data help to explain how ameloblasts respond to the altered extracellular milieu of protons by changing their gene-expression profile throughout the stages of amelogenesis.

Keywords

enamel; mineralization; pH regulation; pH sensing; sodium bicarbonate transporter

Human *SLC4A4* is located on chromosome 4q21 amongst a cluster of enamel and dentin genes, including ameloblastin (*AMBN*), enamelin (*ENAM*), amelotin (*AMTN*), dentin matrix acidic phosphoprotein 1 (*DMP1*), and dentin sialophosphoprotein (*DSPP*) (1). *SLC4A4* codes for the sodium/bicarbonate ($\text{Na}^+/\text{HCO}_3^-$) transporter protein NBCe1, and is essential for the normal development of enamel (1–3). Our previously published data showed that in the ameloblast-like LS8 cell line, the expression of mRNA for the NBCe1-B isoform is pH dependent, with the highest expression seen at a pH of < 7.0 (4). These results showed maximal expression of *NBCe1-B* mRNA at pH 6.0 (4). Since this paper (4) was published, we have repeated the experiment (at pH 6.0, 7.3, and 8.5) using real-time PCR,

and obtained similar results (Fig. 1). Our findings imply that in LS8 cells the *SLC4A4* gene promoter can respond to transcriptional factors in a pH-dependent manner.

Based on published literature it is apparent that no gene promoter analyses have been carried out for the *SLC4A4* gene transcripts (referred to in this manuscript as *NBCe1-A*, *NBCe1-B*, and *NBCe1-C*). The literature is also scant with regards to identifying pH-dependent transcription factors for other bicarbonate transporter genes, of which there are many. One example is the solute carrier *SLC26A4* gene coding for pendrin, an anion exchanger involved in $\text{HCO}_3^-/\text{Cl}^-$ exchange (5). A study on the molecular mechanisms of epithelial-specific expression and gene regulation of pendrin, using a luciferase reporter assay, has identified a DNA element responsive to changing pH within a 96-bp region of the pendrin promoter (5). This 96-bp region is ~ 1,000 bp upstream of the transcription start site (5). For pendrin, this 96-bp pH-responsive region is most responsive in basic conditions, rather than in acidic conditions (5). No other potential pH-dependent transcription factors have been directly associated with this 96-bp region; however, an *in silico* search for possible transcription factors that could bind to this region suggested that the mitogen-activated protein kinase (MAPK) signaling pathway was involved (5).

In the current study we sought to define pH-dependent response elements in the *NBCe1-B* promoter region. Data presented identify potential NFkB1- and potential TP53-binding sites ~ 100–200 bp upstream of the presumed *NBCe1-B* transcriptional start site (TSS). Both NFkB1- and TP53-binding sites are required for reporter gene up-regulation when cells are exposed to acidic pH conditions.

Material and methods

Human *SLC4A4* gene organization

The organization of human *SLC4A4* (coding for the NBCe1 protein) is presented in Fig. 2A. For the *NBCe1-B* variant, the open reading frame begins in exon 2 (Fig. 2). Although no experimental data have established the location of either the TATA box or the TSS for NBCe1, based on the compiled expressed sequence tag (EST) data these sites are predicated by the University of California, Santa Cruz Genome Browser (<http://genome.ucsc.edu/>). The mRNA of the *NBCe1-B* isoform shows an excised intron 1 region. Thus, a presumed (or predicated) TSS is identified here and labeled +1. A 572-bp upstream and 334-bp downstream NBCe1-B DNA sequence directionally cloned into a luciferase reporter assay, referred to here as NBCe1[–572/+334]Luc, was obtained from a commercial source (SwitchGear Genomics, Menlo Park, CA, USA; catalogue #S108221) and used for initial promoter analysis.

Identification of potential pH-responsive elements in the *NBCe1-B* promoter region

An *in silico* analysis was performed on the 572-bp promoter region of *NBCe1-B* in an effort to identify potential transcription factors, and in particular transcription factors and transcription factor-binding sites that may function as pH-responsive elements. The 572-bp *NBCe1-B* promoter sequence, as analyzed by ALGGEN PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) software gated at < 15% dissimilarity, contains potential binding sites for, amongst many others, the gene-transcription factors JUN, NF-κB/NFkB, and NF-κB1/NFkB1 (Fig. 2B) and are of particular interest because all three transcription factors are known to be activated in acidic pH conditions (6, 7). Additionally, ameloblast differentiation is influenced by a pH-dependent JNK signaling pathway (8) and implies that additional transcription factors present in the 572-bp promoter region, including ELK1, member of ETS oncogene family,

SMAD family member 4 (SMAD4), and TP53 may be involved in pH-dependent *NBCe1-B* gene regulation (illustrated in Fig. 2B).

Luciferase-based reporter constructs

Vectors used in this study were the *SLC4A4* promoter (experimental, and referred to here as NBCe1[−572/+334]Luc), the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter (positive control, GAPDH/Luc), and the R_03 promoter (negative control, R_03/Luc) from SwitchGear Genomics (cat. nos: S108221, S121624, and S190003, respectively), and pGL4.23 and pRL-TK (Promega, Madison, WI, USA). All Switchgear vectors and pGL4.23 use the luciferase reporter gene *luc2* (*Photinus pyralis*). Vector pRL-TK uses the alternate luciferase reporter gene *Rluc* (*Renilla reniformis*) that is regulated by the herpes simplex virus thymidine kinase (HSV-TK) promoter.

All NBCe1 DNA fragments identified were generated by a PCR-based approach, which included a *SacI* and an *EcoRV* restriction site in the forward and reverse primers, respectively, and directionally subcloned into pGL4.23 at the *SacI*–*EcoRV* multiple cloning site. These constructs were designated with the general form ‘NBCe1[region contained]^{MinP}Luc’. Within the region of the NBCe1[−292/−8]^{MinP}Luc construct, site-directed mutagenesis was used to engineer nucleotide mutations, as illustrated in Fig. 2C, using the QuickChange Lightning II Site-Directed Mutagenesis System (Agilent Technologies, Santa Clara, CA, USA). All construct inserts used in this study were sequenced in their entirety to ensure that all DNA sequence was as intended.

Ameloblast-like LS8 cells, culture conditions, and methods to adjust culture media to acidic pH for promoter analyses

LS8 cells have been described previously (9–12). Cells were maintained in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered low-glucose Dulbecco’s modified Eagle’s minimum essential medium (DMEM) supplemented with GlutaMAX (Invitrogen, Carlsbad, CA, USA) and 10% fetal bovine serum (FBS). Under normal conditions, HEPES ($pK_a = 7.55$) is sufficient to maintain a constant pH. However, the pH ranges used here were near the limit of its buffer range. Consequently, we chose to use different organic buffer combinations with lower than normal pK_a values. A number of buffers, including MES ($pK_a = 6.15$), Bis-Tris ($pK_a = 6.46$), and piperazine-N,N’-bis(2-ethanesulfonic acid) (PIPES) ($pK_a = 6.80$) allowed for the tight control of environmental pH over the range from physiological pH 7.4 to the most acidic conditions (pH 6.0–6.2) in which these cells can survive (13). To achieve the most effective buffer, we used unbuffered low-glucose DMEM supplemented with 10% FBS, 10 mM PIPES (Sigma Aldrich, St Louis, MO, USA) and 15 mM 3-(N-morpholino) propanesulfonic acid (MOPS) (Fisher Scientific, Pittsburgh, PA, USA), adjusted to pH 6.8. This medium is stable (i.e. it was maintained at pH 6.8) over a 48-h period (our unpublished data), and at this acidic pH there was no apparent change in the growth rate of LS8 cells compared with cells grown at pH 7.4 (Fig. 3).

Transfections and reporter assays

Transfection assays were performed using a standard procedure (9, 14). LS8 cells have been used extensively for transfection studies and have high transfection efficiency (9–11). LS8 cells were seeded into 24-well plates (1.0 – 2.0×10^5 cells per well), and grown to 80% confluence. The cells were then co-transfected with one of the various experimental promoter/luciferase constructs and with Renilla luciferase pRL-TK (Promega) using Lipofectamine 2000 (2 μ l per well; Invitrogen;). Co-transfection with Renilla luciferase [Firefly:Renilla, 1:10 (mole by mole)] compensates for any variations in transfection efficiency (15), and transfections of experimental constructs were performed in triplicate to

compensate for variation among samples. Transfections of control constructs were performed in duplicate. Transfected cells were maintained under normal pH conditions for 24 h, then changed to fresh medium with a pH of 7.4 (control) or 6.8 (experimental) (16) and maintained at these two pH conditions for 24 h.

Luciferase reporter assays were performed using the Dual Luciferase Reporter Kit (Promega; cat. no. E1910) and read on a Turner Biosystems 20/20ⁿ Luminometer (Promega Corporation, Madison, WI, USA). Following transfection and stimulus, the cells were rinsed in PBS and lysed with 100 µl of passive lysis buffer (Promega). The luciferase assays were performed as follows: 10 µl of cell lysate was mixed with 50 µl of Luciferase Assay Substrate (Promega) and read for 10 s twice. Immediately following the second read, 50 µl of Stop 'N' Glo substrate was added, mixed thoroughly, and the sample was again read twice. The relative activity of a given substrate is expressed as a ratio of the average of the two Firefly luciferase readings to the average of the two Renilla luciferase readings. The GAPDH-promoter/luciferase and the β-actin-promoter/luciferase served as positive-control vectors, and a random piece of genomic DNA/luciferase (R_03), of ~ 1 kb, served as a negative control. Experiments were replicated three to five times.

Statistical analysis

Relative activity values for given constructs were collected, and the mean and SD were calculated. To determine significance, the two-tailed Student's *t*-test was performed on each experimental data set. Significance was taken as $P < 0.01$.

Results

The NBCe1[−572/+334]Luc vector showed a significant level of transcriptional up-regulation (> 2.5-fold) in cells maintained for 24 h at pH 6.8 vs. cells maintained at pH 7.4 (Fig. 4, line C). A similar fold change (> 2.1) was noted when this entire NBCe1 DNA region was subcloned into the pGL4.23 vector to create NBCe1[−572/+334]^{MinP}Luc (Fig. 4, line E). Because the pGL4.23 vector backbone contains a minimum promoter (minimum sequence required for efficient transcription), the overall level of transcriptional activity observed from NBCe1[−572/+334]^{MinP}Luc was expectedly higher than seen in the NBCe1[−1057/−152]Luc construct lacking the minimal promoter. In contrast, the GAPDH/Luc positive-control vector gave readable levels of reporter activity (Fig. 4, line B), but there was no significant difference in the transcriptional activity between the two pH conditions used in this study ($P > 0.1$). Another two constructs, R_03/Luc and pGL4.23, were used as negative controls and showed either no or negligible levels of reporter activity under identical test conditions (Fig. 4, lines A and D, respectively).

Based on the *in silico* analysis of potential transcription factor DNA-binding sites contained within the 572-bp promoter region upstream of NBCe1, three islands of potentially pH-responsive regions were identified (Fig. 2B). These three putative pH-responsive regions of DNA were isolated to create overlapping reporter constructs (using the pGL4.23 vector backbone) covering [−572/−445], [−474/−273], and [−292/−8] nucleotides respectively. From these three isolated regions, only the vector NBCe1[−292/−8]^{MinP}Luc (Fig. 4, line H) revealed any significant transcriptional activity, namely a transcriptional up-regulation of > 2.3-fold at pH 6.8 vs. pH 7.4.

Site-directed mutagenesis was used to introduce mutations to the NFκB1-, JUN-, and three TP53-binding sites contained within the [−292/−8] domain (Fig. 2C). From these constructs both the NBκB1- and TP53-binding site mutations resulted in a dramatic decrease in transcriptional activity, although the differences noted at pH 6.8 vs. pH 7.4 were, in most part, statistically insignificant (Fig. 4, lines I, K, L, and M). There was also little change in

the transcriptional activity observed when the JUN site was mutated and assayed by shifting the pH (Fig. 4, line J). These data identify that both the NFKB1 [−188/−177] and TP53 [−114/−91] binding sites are responsive to changes in the extracellular pH, and are most responsive in acidic conditions.

While attention was focused experimentally on NFKB1 and TP53 because they have been implicated in pH-dependent activities, we also used the bioinformatics tool ALGGEN PROMO, gated at < 10% dissimilarity, to reassess the DNA sequences in order to identify other potential transcription factors that may be responsible for the different transcriptional responses noted above. Other transcriptional factors that are predicted as having binding potential at these two DNA regions include; TFAP2A (CGAGGC), NR3C1 (CGAGG and CCTCC), PAX5 (GAGGCC), TP53 (GAGGCC), E2F1 (CTCCCCGC), and MAZ (GCCCCTCCCCGCC) at the NFKB1-binding site [−188/−177]; and NR3C1 (GCAGG), PAX5 (GGGCAG/CG) and ETF1 (GAGGGCAGGGC and GGGCAGGGGGC) at the TP53-binding region [−114/−91]. Additional studies are required to identify which, if any, of these listed transcription factors, is/are responsible for the altered expression profile of the NBCe1 promoter of LS8 cells in acidic media.

Discussion

Enamel mineralization generates large quantities of protons (H^+) (2, 17) and results in an environment driven to increasingly acidic conditions with increasing hydroxyapatite formation (18). Cellular transport processes ensure that the extracellular pH is maintained at levels that are non-toxic to cells, while also being required to allow the continued mineralization of the extracellular matrix to take place. The only cell population in close proximity to the enamel matrix is ameloblasts. Thus, changes in ameloblast gene- and protein-expression profiles of key H^+ /base transporters would be expected to be coordinated with increased extracellular pH buffering that is needed to deal with excess proton release inherent in the mineralization process. The transport of HCO_3^- into the enamel matrix can buffer against extreme extracellular acidity, and NBCe1-B has been identified as a solute carrier in ameloblasts that is capable of mediating this activity (3). NBCe1-B in ameloblasts is up-regulated at both mRNA (4) and protein (3) levels during enamel maturation. The data presented in this study suggest that both NFKB1 and TP53 may be two potent transcription factors involved in changing the NBCe1-B gene-expression profile in response to changes in extracellular pH conditions.

One would expect the fundamental gene-expression activities in enamel biomineralization to be represented across species, such that critical molecular activities identified in one species are also expected to be evident in evolutionary distant species. To address this issue we have aligned the human and mouse genomic regions upstream of the NBCe1-B transcriptional start sites (Fig. 5) with the intention of showing that the mouse NBCe1 promoter is probably under the same influence as the human NBCe1 promoter with respect to a response to changes in extracellular pH conditions. In mouse, the TATA box and TSS are predicted based on multiple published data (19). It is apparent from this alignment (Fig. 5) that both the NFKB1- and TP53-binding sites are conserved between species, and hence may play an identical role in pH-dependent regulation of the *NBCe1-B* gene in mouse ameloblasts. This is thus a testable hypothesis for further investigation.

Finally, our data suggest that transcription of the *NBCe1-B* gene is up-regulated as a result of extracellular acidic conditions, and we have speculated that NFKB1 and TP53 may be gene transcriptional factors involved in such gene transcription up-regulation. How the signal is relayed from the enamel matrix environment to the cell nucleus requires additional studies, and identifying the upstream signaling pathways will be of significance if we are to

understand fully the gene response to an environment of changing pH. While not a topic that has been explored in the mammalian enamel field, the issue of how cells respond to a changing internal and external pH environment is commonly referred to as 'pH sensing'. This clearly is a fruitful area of inquiry that should be addressed in the future to obtain a detailed understanding of how ameloblasts orchestrate the complex changes in H⁺/base transport during the normal synthesis of enamel (20, 21).

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Fig. 1.

Relative expression levels of *NBCe1-B* mRNA at acidic, physiologically neutral, and basic pH conditions assessed by real-time PCR. LS8 cells cultured for 24 h at pH 6.0 showed a 1.41 ± 0.22 -fold (mean $\pm$ SE) increase in *NBCe1-B* mRNA transcripts (using the forward primer 5'-TCCCTGGTATGTGGCTGCTA and the reverse primer 5'-TTGTTCCCTTACTCCCAGAAA; 115-bp product) compared with LS8 cells cultured at neutral pH (pH 7.3), while LS8 cells cultured at pH 8.5 over the same time-period showed a 0.63 ± 0.12 -fold change (mean $\pm$ SE) in *NBCe1-B* mRNA transcripts again compared with cells cultured in neutral-pH conditions. These real-time PCR data were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA profiles (using the forward primer 5'-CATGGCCTTCCGTGTTCCCTA and the reverse primer 5'-GCGGCACGTCAGATCCA; 56-bp product); *GAPDH* mRNA showed no significant fluctuations at the three different pH culture conditions. LS8 cells grown at pH 7.3 (normal pH) showed a variation of $< 1\%$ in *NBCe1-B* mRNA transcript numbers when normalized to the *GAPDH* mRNA control. The reagent used was the iQTM SYBR Supermix from Bio-Rad.

(Hercules, CA, USA). Primer design followed those published on the following website (<http://pga.mgh.harvard.edu/primerbank/index.html>). This experiment was repeated three times, and each time in triplicate. Statistical analysis was performed on the mean values of each of the triplicate experimental set ($n = 3$). The Student's t -test showed significant differences ($P < 0.05$) when the means of low-pH (6.0) and high-pH (8.5) samples were compared with samples grown at pH 7.3.

Fig. 2.

Organization of the human *SLC4A4* (NBCe1-B) gene promoter. (A) Organization of the *NBCe1-B* gene, showing exon 1, exon 2, and the translational start site [open-reading frame (ORF)]. Also shown are the predicted TATA box and the transcriptional start site (TSS). (B) A 572-bp upstream region (TSS = +1) was isolated for promoter studies. The ORF starts in exon 2 (~49.2 kb downstream of exon 1). A very basic restriction map is also shown that identifies unique and strategic cut sites for the regions shown, and the *RsrII* and *XhoI* sites in the upstream sequence are included for orientation of panel B in relation to panel A. Also shown are some predicted transcription factor-binding sites using the *ALGGEN PROMO* software, and regions isolated by PCR for reporter assays, where F1 and R1 represent the forward primer and the reverse primer, respectively, for region 1, F2 and R2 represent the forward primer and the reverse primer, respectively, for region 2, and F3 and R3 represent the forward primer and the reverse primer, respectively, for region 3. (C) The NBCe1-B upstream sequence from [-193/-82], contained within the F3-R3 region, is shown and contains potential NFKB1 (AGGCCCCCTCC), JUN (TGACGCC), or TP53 (GGGCAC/GG) binding sites. Three TP53 sites are present in close proximity. The mutagenesis strategy used to alter these five regions to study gene transcriptional regulation is shown. Dashes represent identities. All images and alignments were performed using *CLUSTAL W* in *MacVector 11.0.4*. NFKB1, nuclear factor of kappa light polypeptide gene enhances in B-cells 1; JUN; jun proto-oncogene; TP53, tumor protein p53.

Growth rates of LS-8 in acidic media

Fig. 3.

Growth rate curves for LS8 cells maintained at pH 7.4 and 6.8. The cells were seeded at 2.0×10^5 cells ml^{-1} and grown for 24 h in HEPES-buffered Dulbecco's modified Eagle's minimum essential medium (DMEM) (pH 7.4). The medium was then changed to either fresh HEPES-buffered DMEM or PIPES/MOPS-buffered DMEM (pH 6.8) and grown for a given period of time. Each time-point is the average number of cells of three plates. No significant differences in growth rate were observed. HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; PIPES; piperazine-N,N'-bis(2-ethanesulfonic acid).

Fig. 4.

Luciferase assay results. LS8 cells were cotransfected with experimental constructs and pRL-TK in triplicate, incubated at pH 7.4 or 6.8 for 24 h, lysed, and assayed for luciferase. The values represent the average raw intensity of a given experimental construct divided by the average raw intensity of the *Renilla* internal standard. These data represent the averages of three to five independent experiments $\pm$ SD. Statistically significant increases are indicated with an asterisk in the 'Fold change' column. The symbol delta (Δ) refers to a deleted region, and hence a mutated construct.

Fig. 5. CLUSTALW alignment of human and mouse promoter region identifying the potential NFKB1- and TP53-binding sites. The positions of human NFKB1 and TP53 are [-188/-177] and [-114/-91], respectively, relative to the transcriptional start site (TSS). The positions of mouse NFKB1 and TP53 are [-172/-163] and [-102/-79], respectively, relative to the TSS. Also identified are the predicted TATA box and TSS (+1) The location of the initial methionine coded by ATG (the start of the open reading frame) is shown immediately after the site of intron 1, here identified at 'N' immediately beneath an asterisk on the bottom line. In humans, *SLC4A4* intron 1 is 49,174 bp in length, and in mice *Slc4a4* intron 1 is 47,424 bp in length. The asterisk in the second bottom line, human and mouse sequences, identifies the most 5' extreme of sequenced mRNAs for both species respectively. The alignment was performed using MacVector 11.0.4. ORF, open reading frame. NFKB1, nuclear factor of kappa light polypeptide gene enhances B-cells1; TP53, tumor protein p53.