Protein-binding elements in the proximal parotid secretory protein gene enhancer essential for salivary-gland-specific expression

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The murine parotid secretory protein (PSP) gene is expressed at high levels in the parotid gland and at lower levels in the sublingual gland. A proximal enhancer core necessary for tissue-specific expression was identified previously, and it was demonstrated that one element, parotid gland element I (PGE I), exhibited specific binding of parotid gland nuclear proteins. In the present study, we demonstrate that a related adjacent element, PGE II, which binds nuclear proteins in a much less tissue-restricted manner, is able to compete with PGE I for binding of parotid-gland-specific factors. The functional significance of PGE I and PGE II was examined in transgenic mice. Deletion

of PGE II reduced transgene expression only in the parotid gland, whereas deletion of PGE I appeared to reduce expression in both of the PSP-expressing salivary glands. Combined deletion of PGE I and PGE II reduced expression below the limit of detection. Thus PGE I and PGE II are functionally important salivary-gland-specific binding elements that are necessary for the salivary-gland-specific expression of a PSP minigene in transgenic mice.

Key words: gene regulation, parotid gland-specific binding elements, PSP, transgenic mice.

INTRODUCTION

Tissue-specific gene expression is a multifaceted process, involving chromatin remodelling, specific binding of transcription factors to cognate DNA elements, and recruitment of large complexes of co-activators/co-repressors in combinations that eventually determine the transcriptional state of a given gene [1,2].

Little is known about the molecular mechanisms ensuring salivary-gland-specific gene expression. A number of genes encoding salivary-gland-specific proteins have been cloned, and studies using transgenic mice have defined several salivary-gland-specific enhancer regions [3]. However, the biological significance of the vast majority of individual salivary-gland-specific binding elements remains to be established. The parotid secretory protein (PSP) gene is one of the most thoroughly characterized salivary-gland-specific genes. PSP mRNA is present at very high levels in parotid gland acinar cells, constituting 10% of the mRNA population, and is present at an approx. 10-fold lower level in the sublingual gland. Transcriptional regulatory regions conferring salivary-gland-specific expression are located in the 5' distal flanking sequences [4–7]. This region includes a 700 bp proximal enhancer core spanning the 5' flanking sequences from –3.6 to –4.3 kb, which is necessary for salivary-gland-specific expression of a PSP minigene [7]. It was shown previously that this enhancer region consists of five elements exhibiting specific binding of salivary gland nuclear proteins and several additional elements capable of binding more ubiquitously expressed nuclear proteins [7]. Based on *in vitro* DNA-binding assays, two novel salivary-gland-specific binding elements, parotid gland element I (PGE I) and sublingual gland element I (SLE I), were considered as prime candidates for principal control regions in the parotid gland and the sublingual gland respectively [7].

In the present study, we present a comprehensive analysis of the functional properties of the prominent binding element PGE

I, and its adjacent and partly homologous element PGE II. By analysis of deletion constructs in transgenic mice, we show that PGE I and PGE II, within the context of the proximal enhancer region, are essential for salivary-gland-specific expression of a PSP minigene.

EXPERIMENTAL

Transgene construction and generation of transgenic mice

The Lama-ΔBsm construction has been described previously [7]. In brief, it contains 4.3 kb of 5' flanking PSP sequences, a PSP-derived transcription unit, and 0.5 kb of PSP 3' flanking sequences. Three Lama-ΔBsm transgenes, ΔBsm-ΔPGE I, ΔBsm-ΔPGE II and ΔBsm-ΔPGE I + II, deleted specifically for PGE I, PGE II or both respectively, were constructed using the transformer site-directed mutagenesis kit (Clontech Laboratories, Inc.), as described by the manufacturer. A pUC19 plasmid containing the proximal PSP enhancer region (–4.6 *Hind*III to –3.1 *Stu*I) [7] inserted between *Hind*III and *Hinc*II was used as the target plasmid [8]. The selection primer changed the *Nde*I restriction site in pUC19 into a unique *Nco*I site. The mutagenic primers were designed to specifically delete the *Nde*I site located between PGE I and PGE II in the PSP insert. Deletion of PGE I (–3577 to –3636) resulted in the sequence junction 5'-GGGACGGCCCC-3', deletion of PGE II generated the sequence junction 5'-ACGGCCGAGACT-3', and deletion of both PGE I and PGE II generated the sequence junction 5'-CCTGGGACGAGACTTCC at the site of mutation. DNase I footprinting was used to demonstrate that protein binding was abolished as intended, and that no artificial protein-binding sites were generated as a result of new sequence junctions created by the deletions. The three different deletion fragments (*Hind*III–*Hinc*II) were subsequently inserted in the Lama vector [4]. The three ΔBsm-ΔPGE constructs were linearized by digestion with

Abbreviations used: PSP, parotid secretory protein; PGE, parotid gland element; SLE, sublingual gland element; DMS, dimethyl sulphate.

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BsmI and *SalI* (Δ Bsm- Δ PGE I and Δ Bsm- Δ PGE I + II) or *BsmI* and *XhoI* (Δ Bsm- Δ PGE II) and injected into the pronuclei of fertilized mouse eggs, as described by Hogan et al. [9]

Identification of transgenic mice

Founder mice and transgenic F₁ mice were identified by PCR analysis of tail DNA. A Lama/PSP specific primer set (primer 1930, 5' GAGGAAGAACAGAAGGATGCC 3'; primer 1931, 5' GGATTTTAATTCTGGGATGGT 3') was used for simultaneous amplification of the region between exon h and exon i in both the transgenic Lama sequence (approx. 0.2 kb) and the endogenous PSP sequence (approx. 1 kb). Southern blotting was subsequently performed in order to establish the intactness of the inserted transgenes, integration patterns and copy numbers [4]. Founder mice were bred [4], and each animal analysed in this study was a progeny of an independent founder or an independent integration event.

Northern blot analysis

Tissue RNA was isolated from the transgenic progeny of founder mice (6–9 weeks of age) using the RNA Isolator kit (Genosys). Total RNA from each tissue was separated by gel electrophoresis and transferred to a nylon membrane. Transgenic mRNA was detected with an oligonucleotide specific for the Lama construct [4]. mRNA expression levels were estimated as described previously [6,7] using a cDNA probe recognizing the transgene as well as the endogenous PSP mRNA [4].

Nuclear extracts and DNA-binding assays

Nuclear extracts from tissues were prepared as reported previously [7], except that the homogenization buffer and sucrose cushions used for preparation of the nuclear extracts contained 0.5 mM dithiothreitol, 0.5 mM PMSF and 1 % protease inhibitor peptide mixture [aprotinin (1 %), leupeptin (1 μ g/ml) and pepstatin (1 μ g/ml)]. Gel retardation assays and DNase I footprinting assays were performed as described previously [7].

Oligonucleotides were purchased from DNA Technology (Science Park Aarhus, Denmark), and the sequences of annealed oligonucleotides were as follows: PGE II, 5' CCATCCTTTTGTCTCCAACAGCCAAGTATTTTCCAT 3' (sense) and 3' GTAGGAAAACAGAGGTTGTCCGGTTCATAAAAGGTAC 5' (antisense); PGE I, 5' GCTAATCCTTAATGCTTCACTGAGATTTTCA 3' (sense) and 3' GATTAGGAATTACGAGTGAACCTCTAAAAGTC 5' (antisense); elastase I element A, 5' TCATGTACCTGTGCTTTTCCCTGCC 3' (sense) and 3' TACAGTGGACACGAAAAGGGACGGAG 5' (antisense); elastase I element B, 5' AGTCAACTCATTTATCTGATA 3' (sense) and 3' AGTTGAGTAAATAGACTATTC 5' (antisense).

Dimethyl sulphate (DMS) protection footprinting

Single-stranded oligonucleotides were 5'-end-labelled with [γ -³²P]ATP using T4 polynucleotide kinase and then annealed to an excess of unlabelled second strand. Gel retardation reactions were assembled as described [7], and bound and unbound DNA were analysed for protection against methylation by the method of Lakin [10].

RESULTS

The PSP proximal enhancer core contains two partly homologous parotid-gland-specific binding elements, PGE I and PGE II

The proximal enhancer core region responsible for salivary-gland-specific expression of the PSP minigene, Lama- Δ Bsm, was

Figure 1 Schematic representation of the two parotid gland elements, PGE I and PGE II

(A) Organization of elements within the proximal core enhancer and downstream flanking sequences. The proximal core enhancer (*BsmI*–*SfiI*) contains five potential salivary-gland-specific binding elements [7]. PGE I is located at the 3' end of the core enhancer, and the partly homologous PGE II is located just 3' of the enhancer core. (B) Fragments used for DNA-binding assays. Synthetic oligonucleotides covering the footprinted regions are designated oPGEI and oPGEII. H, *HinfI*; F, *FokI*; Sf, *SfiI*; S, *StyI*.

shown previously to reside within 700 bp located in the 5' flanking region from –3.6 to –4.3 kb [7] (Figure 1A). PGE I, a novel salivary-gland-specific protein-binding element, is located in this region [7]. DNase I footprinting analysis using the H/S 263 bp fragment (Figure 1B) revealed a very prominent protection of the PGE I region using parotid gland nuclear extracts (Figure 2A, lane 3). DNase I and DMS footprinting analyses also revealed parotid gland protein binding to an adjacent region, PGE II (Figure 2A, lane 3, and Figure 2D). Interestingly, this footprinted region encompasses two sequence motifs, 5' ATTTTCCAT 3' and 5' ATCCTT 3', with high similarity to sequence motifs protected in the PGE I footprinted region (see Figure 4). Gel retardation assays demonstrated that two fragments, F64 and F88 (Figure 1B), covering PGE I and PGE II respectively, were able to compete with one another for protein binding (Figures 2B and 2C). The prevention of DNase I protection and the absence of hypersensitive bands in Figure 2A (lane 5 and 6) demonstrates that the cross-competition patterns were reproduced in a footprinting analysis in which PGE I and PGE II were present on the same fragment, and thus in a context resembling that of the proximal enhancer.

In DNase I footprinting assays, the PGE I oligonucleotide (Figure 1B and Experimental section) prevented/diminished protection of the PGE I region on H/S 263 (Figure 2A, lane 7), and the PGE II oligonucleotide was able to prevent protection of the PGE II region, as indicated by the absence of the hypersensitive site in Figure 2(A) (lane 8). However, the PGE I oligonucleotide was unable to prevent protection of the PGE II region (lane 7), and, similarly, the PGE II oligonucleotide was unable to eliminate protein binding to the PGE I region (lane 8).

Oligonucleotides covering the PGE I and PGE II footprints (Experimental section and Figure 1B) were used in gel retardation and DMS protection assays, and Figure 3 demonstrates the specificity of parotid gland protein binding to both of these oligonucleotides. However, PGE I and PGE II oligonucleotides

Figure 2 Binding of related nuclear proteins to PGE I and PGE II

Competition analyses of parotid gland protein binding to PGE I and PGE II using different enhancer fragments. **(A)** DNase I footprinting analysis of PGE I and PGE II using fragment H/S 263 (lower strand). Samples of 25 μ g of parotid gland nuclear extract (lanes 3–10) were preincubated with the indicated competitor fragments (see Figure 1) present at $\times 100$ molar excess (lanes 4–8) or $\times 200$ molar excess (lanes 9 and 10) prior to the addition of end-labelled H/S 263. Lane 2, control (C) lane without nuclear extract. EIA, elastase enhancer element A; EIB, elastase enhancer element B [12,17]. Lanes 11–16 show the protection profiles of PGE I and PGE II using nuclear extracts (30–40 μ g) isolated from the sublingual gland (SL), submandibular gland (SM), lacrimal gland (LA), spleen (SP), liver (LI) and pancreas (PAN) respectively. **(B)** Competition analysis using the gel retardation assay. Binding of parotid gland nuclear proteins to end-labelled fragment F64 in the presence of increasing amounts ($\times 10$, $\times 50$ and $\times 100$ molar excess) of the indicated competitors. **(C)** Similar competition analysis using fragment F88 in the gel retardation assay. **(D)** DMS footprinting analysis of parotid gland protein protection of guanine residues in the F88 fragment (lower strand). The bound DNA (B) and unbound DNA (F) were separated and isolated as described in the Experimental section, and analysed by denaturing polyacrylamide electrophoresis after being cleaved at methylated guanine residues by piperidine. Cleavages at purine residues are shown in lane G+A.

Figure 3 Binding of related proteins to PGE I and PGE II is dependent on flanking sequences

Parotid gland protein binding to PGE I and PGE II oligonucleotides. **(A)** and **(B)** Gel retardation studies using increasing amounts (molar excess of $\times 50$ or $\times 100$) of competitor fragments. The indicated fragments were preincubated with parotid gland nuclear extract prior to the addition of labelled oligonucleotides. ol, PGE I oligonucleotide; oII, PGE II oligonucleotide. PGE oligonucleotides used as competitors were ligated into concatamers in lanes designated +. **(C)** and **(D)** DMS footprinting of PGE I and PGE II. **(C)** DMS protection footprinting of PGE II using end-labelled PGE II oligonucleotides. **(D)** DMS footprinting analysis of PGE I using end-labelled PGE I oligonucleotides. Two complexes, B1 and B2, were resolved upon electrophoresis of the binding reaction. B, bound DNA; F, unbound DNA. Protected guanidine residues are indicated by arrows. The results are summarized in Figure 4.

were unable to cross-compete efficiently for protein binding in gel retardation assays (Figures 3A and 3B).

Taken together, these results suggest that the protein-binding

sequences of PGE I and PGE II contain a related binding element(s), but that the affinity and/or specificity of parotid gland protein binding to the PGE regions also depends on

Figure 4 Sequence of the region encompassing PGE I and PGE II

PGE I and PGE II sequences protected by parotid gland nuclear extract in the DNase I footprinting assay are indicated by brackets. Partial or weak protection is indicated by broken lines, and arrows indicate the formation of hypersensitive sites upon binding of parotid gland nuclear extracts. Homologous sequences within PGE I and PGE II are shown on a grey background. Hatched boxes indicate sequences containing potential overlapping binding motifs homologous to recognition sites of POU domain and homeodomain transcription factors [17]. Protected guanidine residues within PGE I and PGE II using fragment F88, oPGE I and oPGE II in DMS footprinting assays (Figures 2C, 3C and 3D) are indicated by open triangles.

sequences outside the DNase I-protected regions. Furthermore, these data indicate that the related binding site is composed of homologous motifs situated at the upstream and/or downstream border of both footprints (Figure 4). Thus the PGE I and PGE II regions may contain bipartite binding elements. In sum, these results suggest a functional role for the regions exhibiting sequence similarities between PGE I and PGE II.

PGE I, but not PGE II, is bound by a parotid-gland-specific binding complex

Previously, DNase I footprinting analysis indicated that only parotid gland proteins are able to protect the PGE I element in the context of the H/S 263 bp fragment [7]. The footprinting analysis shown in Figure 5(A) (see also Figure 2A) included nuclear extracts from seven different tissues, and thus provides additional evidence for the parotid-gland-specific protection of PGE I.

Nuclear extracts isolated from the lacrimal gland protected PGE I only weakly. In the gel retardation assay, however, nuclear extracts from different tissues were able to complex with the PGE I oligonucleotide as well as the 64 bp F64 fragment covering the PGE I region, but different proteins appeared to be involved in complex-formation in different tissues (results not shown and Figure 5B).

On the other hand, all nuclear extracts contained proteins capable of interacting with the PGE II region in the footprinting assay, as indicated by the appearance of distinct hypersensitive sites in Figure 2(A) and Figure 5(A) (summarized in Figure 4). Gel retardation assays indicated that complex-formation was common to all tissues investigated, except liver and spleen (Figure 5C).

Footprinting analyses using the two H/S 263 fragments, S170 and S90 (Figure 1), in which PGE I and PGE II are separated, resulted in similar patterns of protection of both PGE I and PGE II compared with those observed using the fragment H/S 263 (results not shown). Thus these experiments exclude the possibility that parotid-gland-specific binding to PGE I is dependent on, or specified by, interactions with PGE II.

To summarize, the results obtained *in vitro* suggest that complex-formation on the H/S 263 fragment, and thus by inference within the proximal enhancer, involves the assembly of a parotid-gland-specific nucleoprotein complex on PGE I, whereas complex-formation on PGE II seems to involve a nuclear complex that shows a broader tissue distribution. Thus, although

Figure 5 Parotid-gland-specific protein binding to PGE I

(A) DNase I footprinting analysis of H/S 263. Nuclear extracts isolated from the parotid gland (PG; 25 μ g), sublingual gland (SL; 40 μ g), submandibular gland (SM; 40 μ g), lacrimal gland (LA; 40 μ g), pancreas (PAN; 40 μ g), liver (LI; 40 μ g) and spleen (SP; 40 μ g) were investigated for their ability to protect PGE I and PGE II. The formation of hypersensitive sites upon binding of nuclear proteins is indicated by arrows. (B) Binding of various nuclear extracts (5 μ g of PA, LA and LI; and 8 μ g of SP, SL, PAN and SM) to fragment F64 in the gel retardation assay. Lanes control to LI were collaged from one gel, and lanes SL to SM from another. The two gels were collaged to present shifts of overall similar intensity using different nuclear extracts. The relative positions of shifts using LA nuclear extracts are similar to shifts obtained using SL nuclear extracts. (C) Gel retardation analysis using end-labelled fragment F88 and 5 μ g of each nuclear extract.

Table 1 Lama- Δ Bsm- Δ PGE transgene mRNA expression in transgenic mice

Transgenic mRNA expression was analysed by Northern blot analysis, and mRNA expression levels in the sublingual glands were quantified in total RNA samples with a PSP cDNA probe. Transgenic mRNA detectable or not by means of Northern analysis is denoted by + or — respectively. Transgenic mRNA expression as a percentage of the endogenous expression of PSP mRNA is indicated in parentheses. Copy numbers and integration patterns were estimated from two independent Southern blot analyses. Transgene head-to-tail (h-t) and head-to-head (h-h) integrations are indicated. *Lama- Δ Bsm was analysed previously [7]. mRNA expression was detected in four out of eight transgenic lines in the sublingual glands and the parotid glands. †Transgenic expression levels in the parotid glands were estimated to be approx. 10-fold below expression levels observed in Lama- Δ Bsm mice. Transgenic hybridization signals from parotid gland mRNA isolated from these mice were detectable by an overnight X-ray exposure, whereas transgenic mRNA signals from all Δ Bsm- Δ PGE lines were undetectable by an overnight exposure using intensifying PhosphorImager screens; 2 weeks of exposure to X-ray films was necessary to detect the transgenic mRNA signals observed in the Northern blots shown in Figure 7(B). ‡Founder mice 1783 and 1786 contained two independent transgenic integrations that were segregated in their progeny. §A very low level of expression in the parotid glands of line 1767 was indicated by the fact that transgenic expression was detectable in only one out of four mice by means of Northern blotting.

Transgenic mouse line	Copy number	Transgenic expression (% of endogenous PSP mRNA level)	
		Parotid (expressing/total lines)	Sublingual (expressing/total lines)
Lama- Δ Bsm*		(4/8)	(4/8)
		+ (< 1%)	+ (10–100%)
Lama- Δ Bsm- Δ PGE I		(2/5)	(1/5)
1784	~ 4–6 (h-t)	—	—
1785	~ 4–6 (h-t)	+ (< 0.1%)†	—
1786–06‡	~ 2–3	—	—
1786–08‡	~ 4–5 (h-t)	+ (< 0.1%)†	—
1787	~ 10–15 (h-t)	—	+ (~ 5%)
Lama- Δ Bsm- Δ PGE II		(2/3)	(2/3)
1767	~ 5–7 (h-t)	+ < 0.1%)§	+ (~ 23%)
1768	~ 5–7 (h-t)	—	+ (~ 110%)
1769	~ 3–5 (h-t)	+ (< 0.1%)†	—
Lama- Δ Bsm- Δ PGE I + II		(0/5)	(0/5)
1779	~ 1–2	—	—
1781	~ 2–4 (h-t)	—	—
1782	~ 1–2	—	—
1783-03‡	~ 2–3 (h-h)	—	—
1783-13‡	~ 1–2	—	—

related or similar nuclear proteins bind PGE I and PGE II, the functional roles of these two partly homologous protein-binding elements are unlikely to be redundant.

Individual deletions of PGE I and PGE II diminish the activity of the proximal enhancer, whereas combined deletion of both elements abolishes salivary-gland-specific expression

To investigate the functional significance of protein binding to PGE I and PGE II, we tested gene constructs with or without these footprinted regions in transgenic mice. Lama- Δ Bsm, which contains 4.3 kb upstream sequence, including the 700 bp proximal enhancer core region, was described and analysed previously [7] (Table 1). PGE I (59 bp) or PGE II (49 bp) was specifically deleted in Bsm- Δ PGE I and Bsm- Δ PGE II respectively, whereas both elements (104 bp) were deleted in Bsm- Δ PGE I + II (Figure 6A).

A total of 13 transgenic lines were identified by PCR (Figure 6B and results not shown). Southern blotting was performed subsequently to characterize integration patterns and copy numbers. The 6.2 and 5.0 kb fragments indicated by the arrows (h-t and h-h respectively) in Figure 6(C) verified the integration of intact transgenes in all strains.

Mice expressing the transgenes were identified by Northern analysis of salivary gland total RNA (Figure 7). mRNA expression levels were estimated using a cDNA probe recognizing the transgene as well as the endogenous PSP gene (described in detail in Laursen and Hjorth [6] and in [7]). As shown in Figures 7(A) and 7(B) and summarized in Table 1, deletion of PGE II reduced parotid gland expression levels relative to those of the Lama- Δ Bsm construct, whereas expression levels in the sub-

lingual glands were comparable with the level of Lama- Δ Bsm expression. The effect of deleting PGE I is shown in Figure 7(B), which suggests that expression levels of the Bsm- Δ PGE I transgene in parotid glands, and possibly the sublingual glands, are significantly reduced relative to those of Lama- Δ Bsm (Table 1). Finally, as seen in Figure 7(B), the combined deletion of PGE I and PGE II reduced transgenic expression to below the detection limit in the parotid glands as well as the sublingual glands (Table 1). Therefore the 104 bp DNA region encompassing PGE I and PGE II, and located between –3636 and –3532, contains sequence information necessary to direct salivary-gland-specific expression of the Lama- Δ Bsm construct.

In conclusion, we were able to detect parotid gland transgene expression in only four out of a total of 13 lines deleted for PGE I and/or PGE II, and expression levels in these lines seemed to be reduced relative to those of Lama- Δ Bsm (Table 1). Out of the 13 lines, only three showed transgene expression in the sublingual glands. Furthermore, transgene expression in the sublingual glands was not predictive for expression in the parotid glands, and vice versa (Table 1). Thus the detected expression patterns for each of the three deletion mutants were not simply the result of chromosomal position effects. Expression in either the parotid glands or the sublingual glands was detected in six out of eight lines deleted for either PGE I or PGE II. However, no expression in either the parotid or the sublingual glands was detected in five lines deleted for both PGE I and PGE II (Table 1).

The effects of the three enhancer deletions in other tissues was analysed by Northern blotting of submandibular gland, lachrymal gland, thyroid gland, heart, liver, spleen, kidney and muscle total RNA from expressing (Tg 1769 and Tg 1786-08) as well as

Figure 6 Transgenic constructs and identification of transgenic mice

(A) Schematic of Lama-ΔBsm gene constructs deleted for PGE I (ΔPGE I), PGE II (ΔPGE II) or both (ΔPGE I + II) by site-directed mutagenesis. See the Experimental section for details. *The Lama-ΔBsm-ΔPGE II construct contains an additional vector fragment of 0.3 kb (the *XhoI*–*HindIII* fragment located at the very 5′ end of the Lama construct) [4]. However, this region has no regulatory effect on Lama expression [4]. (B) Identification of transgenic mice by PCR analysis, as described in the Experimental section. The gel shows the screening of tail DNA from an F₁ litter of founder mouse 1769 (lanes 4–19). Lane 1, positive control (Lama-ΔBsm); lane 2, negative control (non-transgenic); lane 3, negative control without template. The positions of amplification products from the endogenous PSP gene and Lama are indicated. Note that amplification of the transgenic Lama sequence is more efficient than amplification of the endogenous PSP gene (lanes 8 and 16). (C) Southern blot analysis of genomic tail DNA from transgenic mouse lines [4]. *EcoRI*-digested genomic tail DNA was separated by electrophoresis in a 1% (w/v) agarose gel, blotted on to a nitrocellulose filter, and hybridized with a 800 bp probe (PCS) covering PSP sequences from –765 to +30. Migration distances of DNA markers (sizes in kb) are indicated. The expected band sizes of head-to-tail (h-t) and head-to-head (h-h) integration patterns, as well as the hybridization signals of the two endogenous PSP alleles (PSP^a and PSP^b) [4], are indicated by arrows. Different band intensities in different lanes reflect different amounts of loaded DNA. Lane 1, Tg 1787; lane 2, Tg 1786-08; lane 3, 1786-06; lane 4, founder 1786; lane 5, Tg 1785; lane 6, Tg 1784; lane 7, Tg 1783-13; lane 8, Tg 1783-03; lane 9, founder 1783; lane 10, Tg 1782; lane 11, Tg 1781; lane 12, Tg 1779; lane 13, Tg 1769; lane 14, Tg 1768; lane 15, Tg 1767; lane 16, non-transgenic negative control; lane 17, Lama-ΔBsm positive control. Transgenic signals in lane 7 and lane 12 are weak, but the presence of at least one transgene in lines 1783-03 and 1779 has been verified by additional Southern blot analyses.

non-expressing (Tg 1782 and Tg 1783-03) ΔBsm-ΔPGE transgenic lines. Ectopic expression was not observed in any of the four lines examined. Furthermore, no transgenic expression was detected in the submandibular glands in any of the 13 lines (Figure 7C).

Taken together, these functional studies suggest that PGE I and PGE II are partially redundant, positively acting elements. Each element seems to support at least a low level of salivary-gland-specific expression, but both elements are necessary to mediate salivary gland specificity, as well as the enhancer activity of the proximal PSP enhancer. Deletion of positively acting protein-binding elements, and not simply an aberrant enhancer structure, is the most likely explanation for the profound effects of the PGE I and PGE II deletions. Furthermore, the results obtained here strongly indicate that different regulatory mechanisms control expression of the PSP gene in the parotid glands and the sublingual glands.

DISCUSSION

We have evaluated the individual and combined contributions of two protein-binding elements within the PSP proximal enhancer on salivary-gland-specific gene expression in transgenic mice. The effects of specific deletion of *cis*-acting elements have been investigated for other tissue-specific genes, including the mouse elastase I gene and the human α_1 -antitrypsin gene, and several DNA elements and nuclear factors implicated in and/or necessary

for tissue-specific gene expression have been identified [11–13]. This report represents the first study in which the effect of specific disruption of salivary-gland-specific protein-binding elements has been analysed in transgenic mice. We demonstrate that salivary-gland-specific DNA–protein interactions identified for two partly homologous parotid gland elements, PGE I and PGE II, seem to be necessary to specify and activate salivary-gland-specific expression.

The *in vitro* experiments suggest that complex molecular interactions, including parotid-gland-specific factors, are involved in directing the parotid gland specificity of PGE I. The complexes formed with different salivary gland nuclear extracts on the PGE I oligonucleotide are similar to, but clearly different from, those detected with liver and spleen nuclear extracts (P. Svendsen, unpublished work). Strong DNase I protection of PGE I on the larger H/S 263 fragment is only detected with parotid gland nuclear extracts, suggesting the assembly of a salivary-gland-specific nucleoprotein complex in the parotid gland.

PGE II appears to be ‘protected’ in DNase I footprinting assays, irrespective of the tissue used for preparation of the nuclear extracts (Figures 2A and 5A). Furthermore, gel retardation analyses indicate binding of broadly expressed protein(s) to PGE II (Figure 5C).

Sequence similarity between the DNase I footprinted regions of PGE I and PGE II, as well as cross-competition analyses, indicate binding of related or identical nuclear factors to the two

these functional results support evidence gained *in vitro* suggesting that PGE I and PGE II are bound by similar or related nuclear factor(s) in the parotid gland. PGE II is not necessary for high affinity and/or specificity of PGE I protein binding *in vitro*, but the functional data obtained in transgenic mice suggest that the functions of PGE I and PGE II may combine additively for parotid gland specificity and/or cooperatively for enhancer activity.

The differential effects of deleting PGE II in the sublingual glands and parotid glands support previous data suggesting that the PSP gene is regulated differently in the two salivary glands. The functional data suggest that PGE I is, at least partly, involved in directing the sublingual gland specificity of the proximal enhancer. This observation is not immediately corroborated by the results of the DNase I footprinting assays, in which we have been unable to detect binding of sublingual gland nuclear proteins to the PGE I region. Sublingual gland nuclear proteins did, however, bind the F64 fragment, and complex-formation on this fragment with parotid gland and sublingual gland nuclear extracts seemed to be similar or identical. Thus the lack of PGE I protection using sublingual gland nuclear extracts may have been due to some technical failure in detecting sublingual gland complex-formation in the footprinting assay. Alternatively, the PGE I element may play a structural role in the sublingual gland. Sublingual gland expression may be directed by PGE I and/or other salivary-gland-specific binding elements, of which SLE I is a good candidate (Figure 1A). SLE I has been shown previously to represent a unique sublingual DNA-binding element *in vitro*, and proposed to be the prime control element involved in sublingual-gland-specific expression of the PSP gene [7]. The functional data obtained in the present study, however, demonstrate that this element is unable to compensate for the effects of deleting the 104 bp region containing PGE I and PGE II. Thus the activity of SLE I may be dependent on functional interactions within other protein-binding elements, including PGE I, and/or on a precise structural organization of the proximal enhancer.

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REFERENCES

- 1 Struhl, K. (1998) Histone acetylation and transcriptional regulatory mechanisms. *Genes Dev.* **12**, 599–606
- 2 Ogbourne, S. and Antal, T. M. (1998) Transcriptional control and the role of silencers in transcriptional regulation in eukaryotes. *Biochem. J.* **321**, 1–14
- 3 Samuelson, L. C. (1996) Transgenic approach to salivary gland research. *Annu. Rev. Physiol.* **58**, 209–229
- 4 Mikkelsen, T. R., Brandt, J., Larsen, H. J., Larsen, B. B., Poulsen, K., Ingerslev, J., Din, N. and Hjorth, J. P. (1992) Tissue-specific expression in the salivary glands of transgenic mice. *Nucleic Acids Res.* **20**, 2249–2255
- 5 Larsen, H. J., Brodersen, C. and Hjorth, J. P. (1994) High-level salivary gland expression in transgenic mice. *Transgenic Res.* **3**, 311–316
- 6 Laursen, J. and Hjorth, J. P. (1997) *Gene* **198**, 367–372
- 7 Svendsen, P., Laursen, J., Krogh-Pedersen, H. and Hjorth, J. P. (1998) Novel salivary gland specific binding elements located in the PSP proximal enhancer core. *Nucleic Acids Res.* **26**, 2761–2770
- 8 Deng, W. P. and Nickoloff, J. A. (1992) Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. *Anal. Biochem.* **200**, 81–88
- 9 Hogan, B., Constatini, F. and Lacy, E. (1987) *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- 10 Lakin, N. D. (1993) *Transcription Factors: A Practical Approach*, Oxford University Press, Oxford
- 11 Kruse, F., Rose, S. D., Swift, G. H., Hammer, R. E. and MacDonald, R. J. (1993) An endocrine-specific element is an integral component of an exocrine-specific pancreatic enhancer. *Genes Dev.* **7**, 774–786
- 12 Kruse, F., Rose, S. D., Swift, G. H., Hammer, R. E. and MacDonald, R. J. (1995) Cooperation between elements of an organ-specific transcriptional enhancer in animals. *Mol. Cell. Biol.* **15**, 4385–4394
- 13 Tripodi, M., Abbott, C., Vivian, N., Cortese, R. and Lovell-Badge, R. (1991) Disruption of the LF-A1 and LF-B1 sites in the human alpha-1-trypsin gene has a differential effect during development in transgenic mice. *EMBO J.* **10**, 3177–3182
- 14 Komuro, I. and Izumo, S. (1993) Csx: A murine homeobox-containing gene specifically expressed in the developing heart. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8145–8149
- 15 Lints, T. J., Parsons, L. M., Hartley, L. and Harvey, R. P. (1993) Nkx-2.5: a novel homeobox gene expressed in early heart progenitor cells and their myogenic descendants. *Development* **119**, 419–431
- 16 De Jong, R., van der Heijden, J. and Meijlink, F. (1993) DNA-binding specificity of the S8 homeodomain. *Nucleic Acids Res.* **21**, 4711–4720
- 17 Wingender, E., Dietze, P., Karas, H. and Knuppel, R. (1996) TRANSFAC: a database on transcription factors and their DNA binding sites. *Nucleic Acids Res.* **24**, 238–241

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