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GENE EXPRESSION

Identification of cis- and trans-acting factors regulating the expression of rat salivary-specific RP4 gene

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The molecular basis of tissue-specific and cyclic AMP (cAMP)-inducible gene expression in salivary glands is not well understood. Previously, we cloned a salivary-specific proline-rich protein gene, RP4. To analyze the cis-regulatory element(s) that mediates the regulation of this rat salivary RP4 gene, chimeric pRP4CAT constructs containing up to 1.7 kb of the 5′-flanking region of RP4 fused to a reporter gene were transiently transfected into salivary cells. Deletion studies suggest that a 159 bp (−147/+12) fragment of the RP4 5′-flanking region is sufficient to confer salivary-specific induction by agents that can raise intracellular cAMP concentration. Further delineation of this essential sequence revealed that a segment from −136 to −109 is necessary and sufficient to confer cAMP responsiveness in a salivary-specific manner when linked to a heterologous promoter. However, this 28 bp fragment (−136/−109) does not contain an identical match to the consensus cAMP response element (CRE). DNA mobility shift binding assays establish that a sequence-specific DNA–protein complex is formed between this DNA fragment and nuclear proteins from salivary cells, but not with nuclear proteins from HeLa cells, which contain canonical CRE binding proteins (CREBs). Taken together, these data demonstrate that we have identified a 28 bp cis-regulatory element in the RP4 gene that mediates salivary-specific cAMP-inducible gene expression. We propose that the novel salivary-specific CRE binding protein (SCBP) is a key regulator for salivary cAMP-inducible gene expression.

Tissue-specific regulation of gene expression is one of the most challenging research areas in molecular biology. The rat salivary-specific proline-rich protein (PRP) gene, RP4, was chosen to approach this intriguing topic because rat PRPs are expressed exclusively in the rat parotid and submandibular glands. The salivary-specific expression of many closely related rodent PRPs is known to be highly induced upon chronic treatment with β-adrenergic agonists, such as isoproterenol (reviewed by Carlson et al., 1986). However, little is known about the cis-functional elements and their cognate trans-factors that regulate the expression of this family of salivary-specific proteins. Identification of the regulators responsible for this salivary-specific transcription pattern will provide more insight into the processes involved in the development and maintenance of salivary-specific gene expression. Such information would enhance our general understanding of tissue-specific gene expression in mammals. In addition, since many salivary-expressed genes, including the PRPs, are important to oral physiology and health (Mandle, 1987), information regarding the regulation of these genes may have more immediate medical implications.

Cyclic AMP (cAMP) regulates a variety of cel-

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lular processes by activating protein kinase A (PKA) (reviewed by McKnight, 1991), which in turn regulates the expression of various genes by activating nuclear transcription factors. The cAMP response element (CRE)-binding protein (CREB) has been directly implicated in mediating cAMP-inducible transcription of several genes (Lee, 1991; Montminy et al., 1986; Roesler et al., 1988). Furthermore, it has been reported that many other gene promoters contain potential CREB-binding sites and interact with a common transcriptional factor with DNA-binding specificity similar to that of CREB (Hardy and Shenk, 1988; Hyman et al., 1988; Lin and Green, 1988). However, it has not been established that CREB binds efficiently to all of the CREs or that CREB can activate transcription of all the corresponding promoters. Several considerations point to additional cis- and trans-acting factors that are likely to influence CRE function. First, cAMP-induced transcriptional activation is influenced by sequences flanking the CREB binding sites and by other promoter elements (Deutsch et al., 1988; Lee et al., 1989). Second, although several CREs are related by the presence of at least one potential CREB-binding site, their overall structures are distinct (Hyman et al., 1988; Lin and Green, 1988). Third, CREB belongs to a multigene family of activating transcription factors (ATF), with seven or eight members having similar but different DNA-binding specificity (Deutsch et al., 1988). Whether the isoproterenol-inducible expression of RP4 is directly regulated through CRE/CREB recognition, whether other factors are involved, and how this inducibility is related to the tissue-specific expression are important questions to be addressed for understanding PRP gene regulation.

Prerequisites to the elucidation of the mechanism(s) of RP4 gene regulation are identifying the essential cis-regulatory element and assaying its functional contribution under both control and induced conditions. Previously, we cloned and characterized the 5′-flanking sequence of the rat PRP gene, RP4 (Lin and Ann, 1991). In this study, we focus on identifying cis-acting sequence(s) and its putative trans-factors that may be involved in salivary cAMP-inducible gene expression. By using transient transfection assays with a chimeric construct of the cloned 5′-flanking DNA sequence of RP4 gene fused to a reporter gene, chloramphenicol acetyltransferase (CAT), and its 5′ deletion mutants, we localize the regulatory motif(s) that allows for both salivary-specific and cAMP-inducible expression. Furthermore, we identify a 28 bp sequence derived from the RP4 promoter region containing the sequence TGGAGTCA as a salivary-specific CRE. In addition, we identify one protein species from a salivary nuclear extract that binds to this element in vitro. However, no sequence-specific binding can be detected in a HeLa cell nuclear extract, which contains canonical CREBs (Andrisani and Dixon, 1990; Foulkes et al., 1991). Finally, through co-transfection experiments, we show that the overexpression of protein kinase A (PKA) can lead to the induction of the RP4 promoter in salivary cells. Apparently then, rat RP4 gene expression is regulated by a novel trans-regulatory factor(s) that binds to the CRE of RP4 in a salivary-specific manner.

Materials and methods

General methods

All molecular biology procedures were essentially as described by Sambrook et al. (1989) unless otherwise specified. Total RNAs were isolated by lysing cells in guanidinium isothiocyanate according to the method of Chomczynski and Sacchi (1987). All constructs and deletion mutants were confirmed by sequencing analyses using [α-^{35}S]dATP and Sequenase, as described by Chen and Seeburg (1985).

Plasmid construction

Plasmids pBLCAT2 (pTKCAT) and pBLCAT3 (promoterless CAT) were kindly provided by Dr. G. Schütz (Luckow and Schütz, 1987). The pRP4CAT series of plasmids containing up to 1.7 kb of the 5′-flanking region of the rat RP4 gene linked to the CAT reporter gene of pBLCAT3 were constructed by first cloning a Hind III fragment of RP4 gene (−1671/+3185) (Lin and Ann, 1991) into pUC19. After the orientation was determined by restriction mapping and partial sequence analyses, the plasmid was cleaved at the BamH I and Kpn I sites adjacent to 3′ end of the RP4 gene. Subsequently, a nest of inserts unidirectionally deleted, from the 3′ end of the RP4 gene, was generated by exonuclease III and S1 nuclease digestions, as described by Henikoff (1984). After the endpoints of the deletions were determined by sequencing

analyses, the recombinant, pRP4-BK10, containing the insert from −1671 to +12, was selected for further construction. The Hind III/Sac I fragment (blunted by Klenow and S1) of pRP4-BK10 was cloned into the Sma I site of pUC19. Finally, the appropriately oriented clone containing the RP4 5′-flanking region from −1671 to +12 was selected, and after digestion with BamH I/Asp 718 (filled in), was cloned into pBLCAT3 BamH I/Bgl II (filled in). This recombinant is designated as pRP4-1671CAT.

Recombinants pRP4-1311CAT, pRP4-949CAT, pRP4-432CAT, pRP4-247CAT, pRP4-147CAT, and pRP4-110CAT were derived from pRP4-1671CAT by unidirectional 5′→3′ deletion (Henikoff, 1984) of the RP4 5′-flanking sequences. A Pst I/EcoR I(S1-blunted) fragment, corresponding to nucleotides −445/−62 of RP4 (Lin and Ann, 1991), was ligated into blunted BamH I sites in both orientations upstream of the TK promoter in pBLCAT2, and the resulting recombinants were designated as pRP4(−445/−62)TKCAT and pRP4(−62/−445)TKCAT, respectively. Likewise, recombinants pRP4(−250/−62)TKCAT and pRP4(−151/−62)TKCAT were derived from pRP4(−445/−62)TKCAT by unidirectional 5′→3′ deletion of the insert (Henikoff, 1984). Recombinant pRP4(−151/−109)TKCAT was constructed by inserting a PCR-generated Hind III/BamH I-flanked fragment (−151/−109) of RP4 into pBLCAT2; whereas pRP4(−136/−109)TKCAT, pRP4(−109/−136)$_3$TKCAT, and pRP4(−126/−109)$_3$TKCAT were engineered by inserting one copy or three copies of BamH I-flanked synthetic oligonucleotides corresponding to −136/−109 or −126/−109 of RP4 into pBLCAT2.

Cell lines and culture

The rat salivary cell line, RSMT-A5, was kindly provided by Dr. E. E. Kousvelari (Yeh et al., 1988), and passages between 15 and 35 were used in the transfection assays. The Rat-2, CV-1, and HeLa cells were purchased from the American Type Culture Collection. The cells were maintained in either McCoy's 5A medium for RSMT-A5 cells, or Dulbecco's modified Eagle's medium containing 4.5 g/l glucose for the Rat-2, HeLa, and CV-1 cells. In both cases, the media were supplemented with 10% fetal calf serum (FCS), penicillin, 100 units/ml, and streptomycin, 50 μg/ml. Approximately 5×10^5 to 1×10^6 cells were plated per 100 mm diameter dish, and incubated for 24 hours. Two to three hours prior to transfection, the medium was replaced with 10 ml of fresh medium.

Transient cell transfection and enzyme assays

Transient transfections were performed as described previously (Lin et al., 1991) using a standard calcium phosphate coprecipitation procedure. Typically, the amount of transfected DNA was maintained constant (16 μg) by varying the amounts of carrier DNA (pUC19). Three μg of pCH110 (pSV$_2$-β-galactosidase, Pharmacia) were cotransfected as an internal control. Cells were exposed to the precipitate for 5 hours in the presence of 100 μM chloroquine, subjected to a 1-minute 15% glycerol shock, and then washed twice with phosphate-buffered saline (PBS). Twenty-four hours after transfection, the cells were split equally into two 100 mm dishes and maintained in fresh medium with 10% FCS for 36 to 48 hours. Six hours before harvesting, one of the duplicate dishes was treated with 10 μM forskolin.

CAT and β-galactosidase activities were determined as described (Gorman et al., 1982; Herbomel et al., 1984). The transfection efficiencies of the individual transfected cultures were normalized with regard to β-galactosidase activity prior to CAT assay. The expression of CAT was quantitated by cutting out and analyzing the radioactive spots from TLC plates in a scintillation counter. In an effort to ascertain the validity of transfection, the experiments were repeated at least twice with two separate plasmid DNA preparations of the same construct. It was found that the levels of CAT expression among cells transfected independently with separate DNA preparation of the same constructs were highly reproducible, with less than 25% variation between individual transfections.

RNase protection assay

RNase protection assays were performed using a 302-base, ^{32}P-labeled CAT-specific cRNA prepared from pGEM4Z-CAT. RSMT-A5 salivary cells were transiently transfected with different constructs, and total cytoplasmic RNA was prepared (Chomczynski and Sacchi, 1987). Equal amounts of total RNAs (25 μg) were hybridized to the CAT-specific cRNA probe in hybridization buffer (40 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [HEPES], pH 6.4, 400 mM NaCl, 1 mM EDTA, and 80% formamide) overnight at 45°C, and then digested with

RNase A (10 μg/ml) for 1/2 hour at 37°C. After extraction with phenol and precipitation with ethanol, the samples were separated on a 6% urea/polyacrylamide gel. Subsequently, the gel was autoradiographed with two intensifying screens at −80°C for 24 to 36 hours.

Preparation of nuclear extract

Nuclear extracts from RSMT-A5 cells were prepared according to Dignam et al. (1983) and Andrews and Faller (1991). Control and forskolin-stimulated RSMT-A5 cells were suspended in a hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.2 mM phenylmethyl-sulfonyl fluoride [PMSF], incubated for 10 minutes on ice, and vortexed for 10 seconds. Nuclei were collected by centrifugation for 10 seconds at 10,000 rpm, resuspended in 40 μl of cold buffer (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF), and incubated on ice for 20 minutes. Cellular debris was removed by centrifugation, and the supernatant was aliquoted and stored at −80°C. Protein concentration of the nuclear extract was determined (Bio-Rad protein assay kit).

Gel mobility shift assays

A 28 bp oligonucleotide corresponding to −136/−109 in the RP4 promoter (Lin and Ann, 1991) was used as a probe. Nuclear extracts (10 μg) were incubated with 5,000 cpm of the ^{32}P-labeled DNA probe for 45 minutes at room temperature in the presence of 10 mM Tris HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 4 μg of poly(dI-dC)·poly(dI-dC), 1 μg of denatured salmon sperm DNA, and 5% glycerol in a final volume of 20 μl (Durand et al., 1988). The reaction mixture was electrophoresed in a 10% polyacrylamide gel (30:1 acrylamide: bisacrylamide) with 0.5X TBE buffer (45 mM Tris-borate, 45 mM boric acid, 1 mM EDTA, pH 8.3) at 4°C. The gel was dried and autoradiographed at −80°C for 4 hours.

Results

Functional analysis of the RP4 promoter

To identify specific region(s) that regulate the expression of the rat salivary-specific PRP gene, RP4, we transfected rat salivary cell lines, RSMT-A5, with plasmids containing different 5′ regions of the RP4 gene inserted upstream of a CAT reporter gene. In all experiments presented in this report, transfection efficiency was normalized by cotransfecting plasmid pCH110, which contains the β-galactosidase gene under the control of the SV40 early promoter (Herbomel et al., 1984; see Materials and Methods for details). The parental plasmid for this series of construction was pRP4-1671CAT, which contains RP4 sequences −1671/+12, where the RNA start site is designated +1 (Lin and Ann, 1991). A series of constructs was prepared using exonuclease III and S1 nuclease to make progressive 5′ deletions in the pRP4-1671CAT. These constructs had common 3′ ends (+12), and 5′ ends at −1311, −949, −432, −247, −147, or −110, respectively. The results of a representative transfection in the salivary cells are shown in Figure 1A, in which deletion of sequences between −1672 and −432 had little effect on the expressed CAT activities. Transfection with pRP4-147CAT, however, produced the highest CAT activity (Fig. 1A, lane 6) suggesting there may be a negative regulatory element 5′ upstream from nucleotide −147. Moreover, a large reduction in CAT activity resulted from deletion of nucleotides −147/−110 (Fig. 1A, lanes 6 and 7). Recombinants with additional 5′ deletions have CAT activities close to that of promoterless pBLCAT3 (data not shown); therefore, it appears that the recombinant pRP4-110CAT contains the basal promoter for RP4 gene expression. This deletion experiment also suggested that a potential positive regulatory element is located within nucleotides −147/−110.

Differential and induced expression of pRP4-147CAT in cultured cells

Comparison of RSMT-A5 cells transfected with the series of pRP4CAT deletion mutants with or without stimulation by forskolin, an adenyl cyclase agonist (Seamon et al., 1981), reveals that pRP4-147CAT not only confers the basal activity but also results in the highest inducibility (Fig. 1B). Therefore, pRP4-147CAT was further transfected into both salivary cells (RSMT-A5) and nonsalivary-derived cells (HeLa, Rat-2, and CV-1) to assess the responsiveness of pRP4-147CAT to various pharmacological stimulants. As shown in Figure 2, treatment of transfected salivary cells with 10 μM isoproterenol plus 0.5 mM theophylline (lane 3), 10 μM isoproterenol

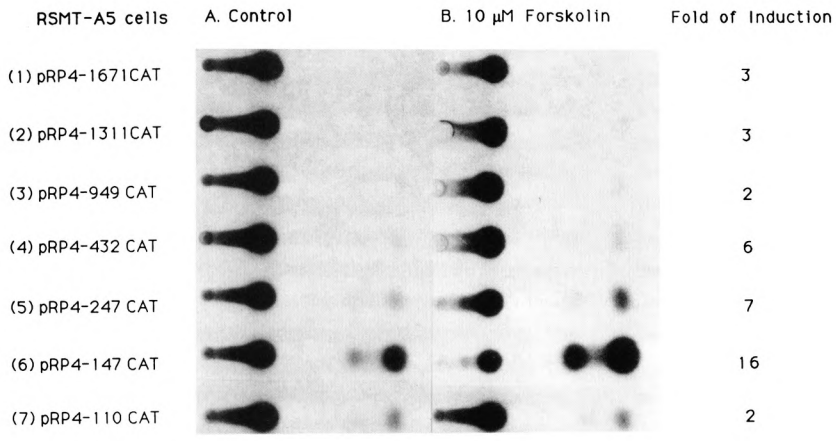

Figure 1. Functional analysis of the RP4 promoters. pRP4-1671 CAT and its deletion mutants were constructed as described in Materials and Methods. Rat salivary RSMT-A5 cells were co-transfected with equal amounts, on a molar basis, of each pRP4-CAT fusion gene, as indicated, and 3 μg of the reporter expression vector, pCH110. CAT assays were performed with equal amount of protein extracts, after normalization with β-galactosidase expression (see Materials and Methods for details). Cells were (A) control, or (B) treated with 10 μM forskolin for 6 hours prior to harvest. Acetylated products of chloramphenicol are displayed following thin-layer chromatography and autoradiography. Relative fold of inductions was calculated from % conversion of cells treated with forskolin over that of control cells.

alone (lane 4), or 10 μM forskolin (lane 6) led to a significant activation of CAT activities relative to that of the untreated cells (lane 2). However, pretreatment of transfected cells with the β-adrenergic antagonist, propanolol (50 μM), for 15 minutes completely blocked the observed CAT induction by isoproterenol (lanes 4 and 5). Theophylline alone (lane 1) did not significantly activate CAT expression but did enhance the stimulatory effect of isoproterenol (lanes 3 and 4).

Since forskolin increases intracellular cAMP levels (Seamon et al., 1991), and the effect of

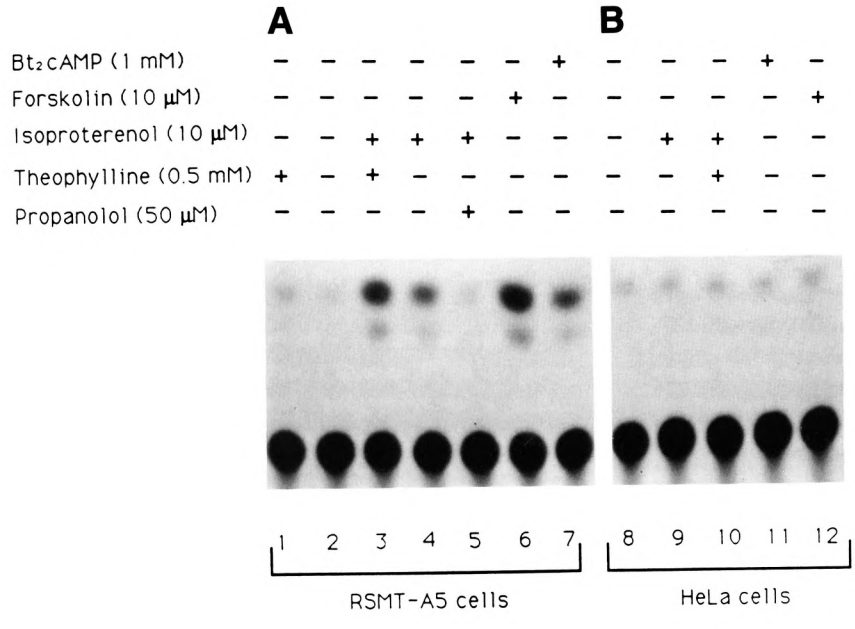

Figure 2. Pharmacological effects on transient expression of pRP4-147CAT in salivary and HeLa cells. RSMT-A5 cells (**A**) and HeLa cells (**B**), which had been transiently transfected with pRP4-147CAT, were incubated for 6 hours with isoproterenol (10 μM), dibutyryl cAMP (1 mM), theophylline (0.5 mM), or forskolin (10 μM), or combinations thereof. When used, propanolol (50 μM) was added to the cells for 15 minutes prior to the 6-hour incubation periods. Subsequently, the cells were harvested, and cellular protein, normalized for β-galactosidase activity, was analyzed for CAT activity. Acetylated products of chloramphenicol are displayed following thin-layer chromatography and autoradiography. Bt_2cAMP: dibutyryl cAMP.

isoproterenol on CAT induction was inhibited by propanolol, it is suggested that the effect of isoproterenol on the transfected RSMT-A5 cells is mediated by the β-adrenergic receptor with cAMP as the second messenger. This is demonstrated in lane 7 of Figure 2, as the addition of 1 mM dibutyryl cAMP increased CAT activity significantly over the basal level. Similar experiments were also carried out on other cultured cells, such as HeLa (Fig. 2), Rat-2, and CV-1 (data not shown). The basal level of CAT activity in the transfected HeLa cells was comparable to that of the RSMT-A5 cells (lanes 2 and 8). However, following treatment with any of the agents, no obvious induction of CAT activity was observed in transfected HeLa and other nonsalivary cells (Fig. 2, lanes 9 to 12). The results presented in Figure 2 imply that the differences in induction by pharmacological agents between salivary and nonsalivary cells transfected with pRP4-147CAT may be due to distinct regulatory factors in various cells that modulate the expression of a given DNA responsive element differently. It also suggests that the rat salivary cell line, RSMT-A5, possesses the necessary transcription factors for the salivary-specific induction of PRP gene expression, as was observed in vivo (Ann et al., 1987). Since there was no significant difference between isoproterenol and forskolin treatment in terms of the induction of pRP4-147CAT expression in transfected RSMT-A5 cells, and both isoproterenol and forskolin exert their pharmacological effects through cAMP, transfected cells were treated with 10 μM forskolin only throughout the rest of the presented studies.

Transcriptional control of pRP4CAT constructs by forskolin

One goal of our studies is to use a functional assay to localize the regulatory element that governs the salivary-specific, cAMP-inducible expression of the RP4 gene. As shown in Figure 1, there is very little or no induction in CAT activities upon forskolin treatment of cells transiently transfected with the recombinant constructs pRP4-1671CAT, pRP4-1311CAT, and pRP4-949CAT. However, forskolin treatment of cells transfected with pRP4-147CAT resulted in a 16-fold induction in CAT activity (Fig. 1, lane 6). Cells transfected with a construct with a further 5′→3′ deletion, pRP4-110CAT, were dramatically less sensitive to forskolin in terms of induction of CAT activity (Fig. 1, lane 7). These results suggest that a potential CRE is located within nucleotides −147/−110 of the RP4 gene. This potential CRE coincides with the proposed positive regulatory element, which is consistent with the notion that the CRE can also function as a basal enhancer (Roesler et al., 1988).

To ensure that the observed inductions of CAT activities truly reflect the changes in CAT mRNA levels rather than a modulation of protein synthesis or enzymatic activity by cAMP, transfectants were analyzed by RNase protection (Fig. 3). In agreement with the CAT activities presented in Figure 1, cells transfected with the recombinant pRP4-147CAT demonstrated the highest basal and cAMP-induced CAT message levels among the three transfectants analyzed, as shown in Figure 3. This observed induction in CAT mRNA following forskolin treatment substantiates the hypothesis that the regulation of rat RP4 gene expression involves activation at the transcriptional level. The up-regulated basal CAT messages of pRP4-147CAT in lane 7 compared to levels in lanes 3 and 5, suggest that there is a putative negative regulator located between nucleotides −432 and −147 of the RP4 gene, which is consistent with observations concerning the transient transfection assays in Figure 1. These results also suggest that the basal and cAMP-induced expression of pRP4CAT constructs is repressed by this putative negative regulatory element, either directly or indirectly in our assay system. It is likely that sequences upstream of −1671 or downstream of +12 might be essential for overcoming this negative control. Nevertheless, these deletion analyses clearly demonstrate that there is a putative positive regulatory element residing between −147 and +12.

Induction of pRP4TKCAT constructs in various cell lines

In order to assess whether the 5′-flanking region of RP4 would confer cAMP inducibility upon a heterologous promoter (HSV TK) in different host cells, the −445/−62 fragment of RP4 was inserted 5′ of the TK promoter of pBLCAT2 in both orientations, pRP4(−445/−62)TKCAT and pRP4(−62/−445)TKCAT, respectively. Two 5′→3′ deletants, pRP4(−250/−62)TKCAT and pRP4(−151/−62)TKCAT, were derived from pRP4(−445/−62)TKCAT. The results of transient transfections with this series of con-

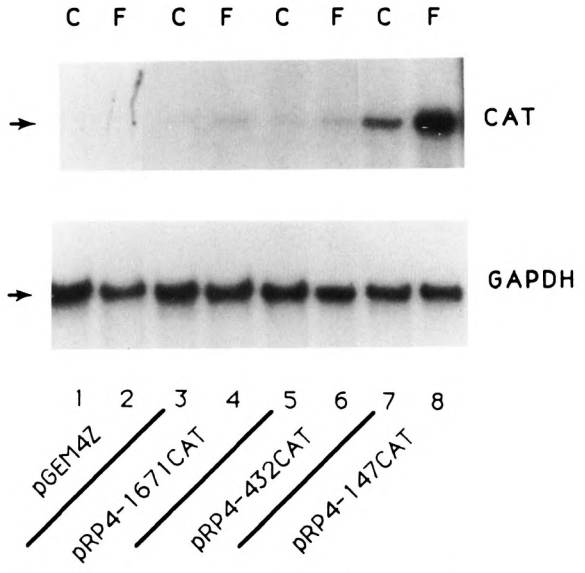

Figure 3. Transcriptional activation of pRP4CAT constructs in transiently transfected salivary cells. Total RNAs were isolated from RSMT-A5 cells transfected with pRP4-1671CAT (lanes 3 and 4), pRP4-432CAT (lanes 5 and 6), pRP4-147CAT (lanes 7 and 8), or pGEM4Z (lanes 1 and 2, as a negative control), respectively. To quantitate CAT mRNA, 20 μg of total RNAs from each of control (C) and forskolin-treated (F) sample were hybridized with a CAT-specific cRNA probe and analyzed by RNase-protection analysis. The protected CAT fragments are indicated by an arrow. The same preparations of RNAs were also analyzed with a specific glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cRNA probe to demonstrate the integrity of RNA preparations and to serve as an internal control.

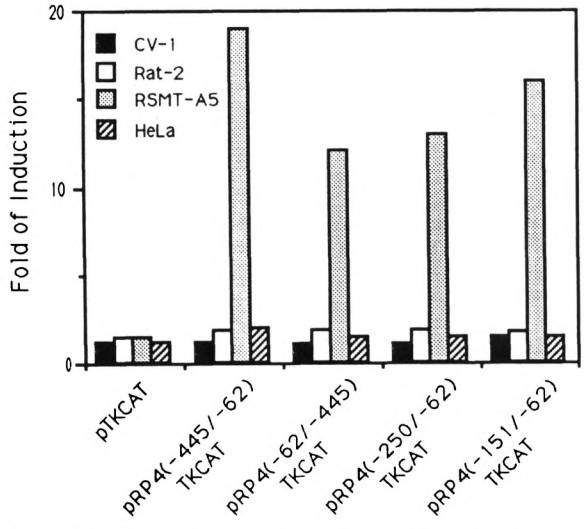

Figure 4. Quantitative comparison of cell-type dependent induction of CAT activity by cAMP in cells transfected with pRP4TKCAT constructs. The 384 base pair region of RP4 from −445 to −62 was inserted in front of the TK promoter in the sense (pRP4(−445/−62) TKCAT) and reverse (pRP4(−62/−445)TKCAT) orientations. The 189 base pair (−250/−62) and 90 base pair (−151/−62) regions of RP4 were inserted in front of the TK promoter only in the sense orientation, and designated pRP4(−250/−62)TKCAT and pRP4(−151/−62) TKCAT, respectively. pTKCAT was used as a control. CAT assays were performed on cells transiently transfected with the indicated plasmids. The fold of induction expressed in CV-1 (■), Rat-2 (□), RSMT-A5 (▨), and HeLa (▨) cells is calculated by dividing the CAT activity in extracts from cells stimulated with forskolin for 6 hours prior to harvest by that from the control cells, after normalizing with β-galactosidase activity. Each value represents the mean of at least three separate transfection experiments, and the variation from the mean was generally less than 25%.

structs are shown in Figure 4. The region of RP4 covered by the smallest fragment tested (−151/−62) is nearly contained within the pRP4–147CAT(−147/+12) construct, which gave the highest basal level and fold of induction by cAMP when transfected into salivary cells (Fig. 1). Each of the four constructs demonstrated cAMP inducibility only when transfected into RSMT-A5 salivary cells, whereas no induction was observed in CV-1, Rat-2, and HeLa cells (Fig. 4). Taken together, the results presented in Figure 2 and this study, in which RP4 and TK promoters were used, suggest that the DNA fragment able to confer cAMP inducibility on both homologous and heterologous promoters is salivary-specific. However, the negative regulatory element demonstrated in the pRP4CAT deletion experiments (Fig. 1) has little or no effect on the cAMP responsiveness of the heterologous TK promoter (Fig. 4) and thus appears to be RP4 promoter-specific.

To delineate further the minimal sequence necessary for this observed cAMP-dependent expression, a segment of the RP4 gene extending from −151 to −109 was linked to TKCAT (pRP4(−151/−109)TKCAT) and tested for inducibility of CAT activity by forskolin. As shown in Figure 5, when this 43 bp sequence was linked to the TK promoter and transiently transfected into salivary cells, CAT activity was significantly induced by cAMP (~13-fold). Analysis of this 43 bp sequence reveals the presence of bases TGGAGTCA (−117 to −110), identical in 6 of 8 bases to the consensus CRE, TGACGTCA, which has been implicated in the cAMP responsiveness of several genes (Roesler et al., 1988). To determine the importance of this sequence in conferring cAMP regulation to the RP4 gene,

we introduced three tandem copies of the −126/−109 fragment in front of the TK promoter, pRP4(−126/−109)$_3$TKCAT. When this construct was transiently transfected in salivary cells, a modest cAMP inducibility (~3-fold) was reproducibly observed (Fig. 5). Thus, this region alone (−126 to −109), although containing the 8-base pair potential CRE homologue, is not sufficient for the observed strong cAMP inducibility of RP4 gene expression. However, when an extra 5′ end 10 bp were included in the pRP4TKCAT construct, pRP4(−136/−109)TKCAT and pRP4(−109/−136)$_3$TKCAT, a moderate (~6-fold) and a strong (~11-fold) induction, respectively, of CAT activities by forskolin treatment were reproducibly observed (Fig. 5). The increase in copy numbers from one to three almost doubles the fold of induction by forskolin, which is consistent with other researchers' findings (Ondek et al., 1987; Schaffner et al., 1988). The extra 5′ end 10 bp may not be by itself sufficient for the observed salivary cAMP inducibility; rather, we speculate that more than one nuclear factor may be involved. This is similarly reported in the case of pancreas acinar cell-specific transcription factor that contains two subunits and recognizes bipartite motifs (Roux et al., 1989). The lack of constitutive enhancement activity from the 43 bp (−151/−109) or 28 bp (−136/−109) fragments on the TK promoter (Fig. 5) may be because the basal enhancer activity of this CRE is RP4 promoter-specific, as has been reported for other CREs (Kanei-Ishii and Ishii, 1989).

Identification of nuclear proteins that bind to salivary CRE of RP4

To investigate the possible interaction of nuclear proteins with the regulator element we have identified and its importance to RP4 gene expression, sequence-specific binding was studied through a gel mobility shift assay. As shown in Figure 6, when probe A, extending from −136 to −109 of the RP4 gene, was incubated with nuclear extracts from either normal or forskolin stimulated salivary cells and subjected to electrophoresis in a nondenaturing polyacrylamide gel, one major species (indicated by an arrow) was detected (Fig. 6, lanes 2 and 3). No significant difference was observed in terms of mobility, size, or number of complexes formed between the nuclear proteins prepared from

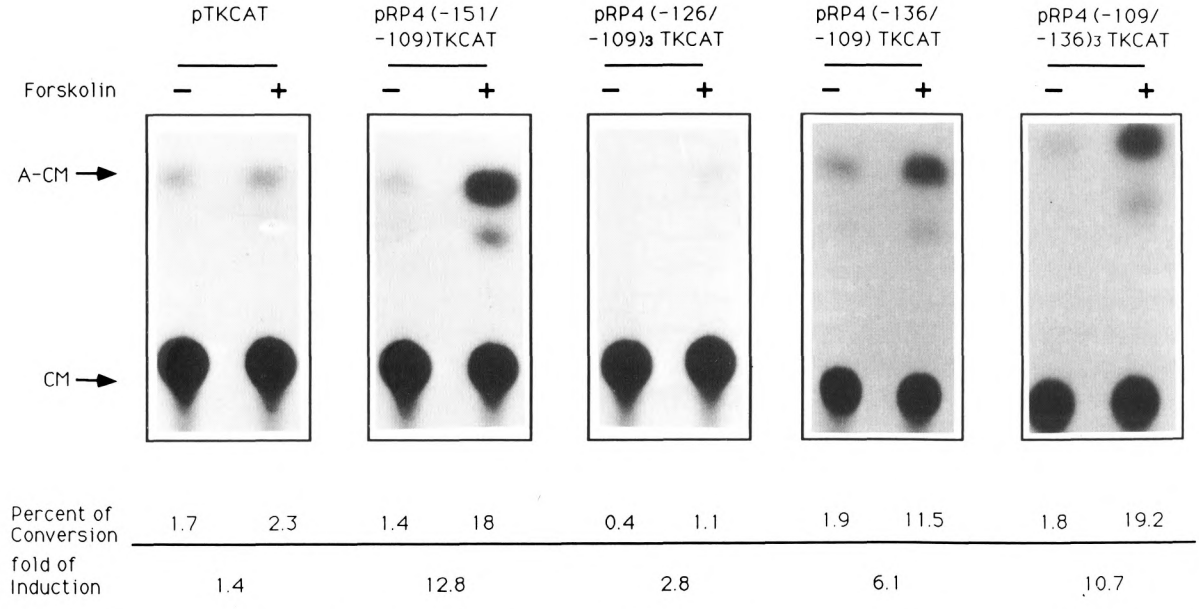

Figure 5. Delineation of the minimal sequences from the RP4 5′-flanking region that confer cAMP regulation to a heterologous promoter. Salivary cells were transfected with pTKCAT, pRP4(−151/−109)TKCAT, pRP4(−126/−109)$_3$ TKCAT, pRP4(−136/−109)TKCAT, or pRP4(−109/−136)$_3$TKCAT and cultured in the absence (−) or presence (+) of 10 μM forskolin. Equal amounts (normalized) of cell extracts were assayed for CAT activities. Acetylated products of chloramphenicol are displayed following thin-layer chromatography and autoradiography. The calculated percent of conversion and fold of induction are shown.

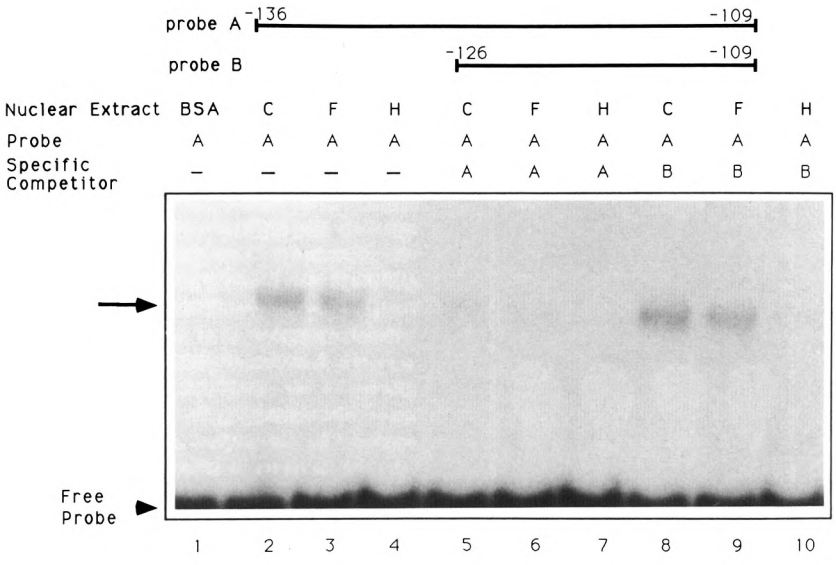

Figure 6. Identification of nuclear factor(s) that binds to RP4 gene fragments from salivary cells. The gel mobility shift assay was performed as described in Materials and Methods with either 10 μg of nuclear extracts from control (C), forskolin-treated (F) salivary cells, HeLa cells (H), or 10 μg of bovine serum albumin (BSA). The locations of the probes and competitors in the 5′-flanking region of the RP4 gene used in the gel mobility shift assay are indicated above. In the competition experiment, 100-fold molar excess of unlabeled probes A and B were used as sequence-specific competitors. The sequence-specific complexes are indicated by an arrow.

control and forskolin-treated salivary cells (Fig. 6, lanes 2 and 3). The addition of excess unlabeled probe A (−136/−109) resulted in the disappearance of the salivary complexes (Fig. 6, lanes 5 and 6; indicated by an arrow). Thus, these complexes represent sequence-specific DNA–protein complexes. Similar gel mobility shift and competition experiments were carried out with nuclear proteins from HeLa cells, which are known to have canonical CREBs in their nuclear extracts (Andrisani and Dixon, 1990; Foulkes et al., 1991). However, no complex was detected when the same amount of nuclear protein from HeLa cells was used (Fig. 6, lane 4). This substantiates our previous observation (Figs. 2 and 4) that the cAMP responsiveness of RP4 is salivary-specific.

To further localize the recognition site of the binding activity, probe B (−126/−109), which is contained within the 3′ end of probe A and contains the 8 bp CRE-homologous sequence, was used as a competitor. However, probe B (−126/−109) neither affected the formation of salivary complexes with probe A (−136/−109), since no competition was observed (Fig. 6, lanes 2 and 3 versus lanes 8 and 9), nor formed sequence-specific complex when incubated with nuclear extracts from salivary cells (data not shown). Therefore, this indicates that probe B alone is not sufficient and that the 5′ end of probe A is critical for complex formation with salivary nuclear proteins. This is consistent with our observation that three tandem copies of the (−126/−109) fragment, despite the potential homology of the 8 bp sequence contained within this fragment with the consensus CRE, conferred only a modest cAMP responsiveness (3-fold) to the TK promoter in salivary cells (pRP4(−126/−109)$_3$TKCAT; Fig. 5). From the results of the gel mobility shift (Fig. 6) and transfection (Fig. 5) assays, we conclude that the salivary-specific CRE of the RP4 gene resides in the 28 bp segment from −136 to −109, and this CRE interacts with (a) constitutive salivary-specific factor(s) to control RP4 gene expression.

Activation of RP4 CREB by overexpressing PKA

Having observed that the complexes formed with nuclear extracts from either control or forskolin-treated salivary cells in the gel mobility shift assay are almost identical in quantity and size (Fig. 6, lanes 2 and 3), we examined the possible involvement of PKA in the cAMP induction of RP4 gene expression. The salivary cells were either transfected alone with pRP4 (−151/−109)TKCAT or cotransfected with pRP4 (−151/−109)TKCAT and an expression vector encoding the catalytic subunit of PKA (Maldonado and Hanks, 1988). All the transfections included a constant amount of β-galactosidase expression vector to monitor transfection efficiency. As shown in Figure 7, the overexpression of PKA stimulated the CAT activity as much as did forskolin, supporting the hypothesis that salivary cells have (a) pre-existing PKA-responsive transcription factor(s). The combination of overexpressing PKA and forskolin treatment

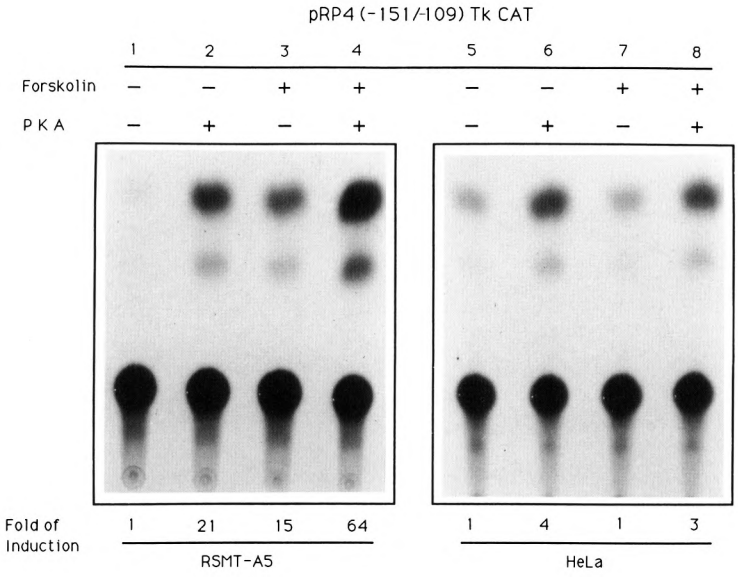

Figure 7. Effect of overexpression of PKA on RP4 CRE construct in salivary and HeLa cells. Salivary and HeLa cells were cotransfected with pRP4(−151/−109)TKCAT (5 μg), and pCH110 (2 μg), with or without 10 μg of the PKA expression vector, pSK-G4 (pSV$_2$-PKA; Maldonado and Hanks, 1988). Subsequently, the cultures were incubated in the absence (−) or presence (+) of 10 μM forskolin for 6 hours. Equal amounts (normalized) of cell extracts from individual samples were assayed for CAT activity. Acetylated products of chloramphenicol are displayed following thin-layer chromatography and autoradiography. The calculated fold of induction of CAT activity is also shown.

(Fig. 7, lane 4) resulted in a response that was significantly greater than either PKA or forskolin treatment alone, suggesting that PKA and forskolin may act by a synergistic mechanism. We thus attribute the cAMP responsiveness of salivary cells to a constitutive CREB in these cells, which can be activated by PKA and subsequently mediates induction of RP4 gene expression. Identical experiments were also carried out in HeLa cells. Despite the fact that a comparable basal level expression of pRP4 (−151/−109)TKCAT was seen in HeLa cells, which are known to have canonical CREBs, the overexpression of PKA alone resulted only in a weak activation (4-fold versus 21-fold) of CAT expression, and no effect of forskolin treatment was observed (Fig. 7). This is consistent with the notion that this element is salivary-specific (Figs. 2, 4, and 6). Similar results were also obtained with construct pRP4(−109/−136)$_3$TKCAT (data not shown).

Discussion

We have identified a minimal sequence, extending from −136 to −109 of the RP4 gene, which is capable of conferring cAMP regulation to a heterologous promoter (TK) specifically in salivary cells. Also identified is constitutively expressed salivary nuclear factor(s) which binds to this fragment and, upon activation by either PKA or forskolin, triggers RP4 gene expression. This represents the first functional delineation of a salivary CRE and its binding factors for the rat salivary-specific proline-rich protein multigene family. We propose the designation of SCBP (*s*alivary-specific and *c*AMP-responsive element *b*inding *p*rotein) for this salivary factor until its nature is further defined.

In general, cAMP inducibility at the level of transcription is mediated by a CRE in the promoter of the target gene. However, the context where the CRE resides also plays an important role in its ability to confer cAMP inducibility (Kanei-Ishii and Ishii, 1989). Evidence has now accumulated that CREs can have different functions. Some CREs serve as enhancers without conferring responsiveness to cAMP, as in the case of the HTLV-1 LTR and Hsp70 promoters (Hai et al., 1989). Some CREs are responsible for mediating the inducibility by both cAMP and by calcium influx, as in the case of the somatostatin gene (Montminy et al., 1986). Others can even mediate repression of gene transcription (Boshart et al., 1990). Here, we report a CRE that serves as a constitutive transcription activator and confers cAMP responsiveness on both homologous and heterologous promoters; however, these functions of this novel CRE can be observed only in salivary cells.

The precise mechanism governing the salivary-specific PRP gene regulation remains to be confirmed. One hypothesis is that there is a different tissue distribution of its regulatory factors. This has been previously noted for transcription factors from the ATF family, the ex-

pression of which differs in various cell types (Park et al., 1990). A more complex scheme may be proposed, involving factors such as the recently reported CRE modulator (CREM; Foulkes et al., 1991 and 1992). Following a developmental switch, different isoforms of CREM, resulting from alternative splicing of one gene, function either as transcriptional agonists or antagonists in response to cAMP (Foulkes et al., 1992). Thus, the presence of one or more isoforms of CREM in some cell types interacting with this CRE could also explain the lack of cAMP inducibility of the RP4 gene in other cell types.

The lack of significant differences in the size and number of complexes formed between RP4 CRE and nuclear extracts of control and forskolin-treated salivary cells may be explained by the nature of this cAMP-regulated gene expression, in that the cAMP-regulated gene induction is achieved by phosphorylation of a constitutive nuclear factor, CREB (Meinkoth et al., 1991; Rehfuss et al., 1991; Riabowol et al., 1988). This may become a paradigm for the phenomenon of cAMP-regulated gene expression as reported by us here and by others (Kagawa and Waterman, 1991; Lamph et al., 1990). A completely homologous CRE consensus sequence, TGACGTCA (Roesler et al., 1988), and AP-2 binding sequence, CCCCAGGC (Imagawa et al., 1987), are not found in the RP4 5′-flanking sequence; however this has been observed previously for another cAMP-regulated gene, $p450_{c21}$ (Kagawa and Waterman, 1991). Despite this lack of homology, the salivary-specific cAMP-responsive element in the rat RP4 gene has been concurrently localized by analyses of CAT activities and RNA transcripts (Figs. 1 and 3) and through the use of a heterologous promoter (Figs. 4 and 5).

The segment of the rat RP4 gene containing the identified salivary-specific CRE (nt −136 to −109) is somewhat conserved at approximately the same position among known mouse and rat PRP genes (Ann and Carlson, 1985; Lin and Ann, 1991; Roberts et al., 1991; Fig. 8). Roberts et al. (1991) reported that the promoter region −141/−111 of mouse PRP gene, which is homologous to the region we have identified (Fig. 8), forms complexes with nuclear extracts from isoproterenol-induced mouse parotid glands and also from HeLa cells, but not with that from control salivary glands, isoproterenol-induced submandibular glands, or other mouse tissues. In contrast, we have repeatedly observed the same complexes formed, using nuclear extracts prepared from control and forskolin induced salivary cells, but not from HeLa cells. The reasons for this observed discrepancy between the two homologous promoter regions (Roberts et al., 1991, and this report) are not clear at present. Possible explanations are (1) genetic divergence between mice and rats, (2) the different pharmacological effects of short-term (6-hour) induction versus chronic treatment, and (3) differences arising from treatment with forskolin in vitro versus treatment with isoproterenol in vivo. Chronic isoproterenol treatment of rodents not only induces PRP gene expression but also stimulates hyperplasia and hypertrophia of salivary glands (Schneyer, 1972 and references therein). The mechanisms by which isoproterenol functions as a mitogen in the salivary gland and induces PRP gene expression in vivo are still unclear. In our in vitro salivary cell system, the regulation of RP4 gene expression by various pharmacological agents (Fig. 2) suggests a possible mechanism to account for isoproterenol-induced PRP gene expression. Pretreatment of salivary cells with propanolol and treatment with dibutyryl cAMP inhibit and mimic the effect of isoproterenol, respectively, indicating that cAMP is the second messenger for this effect of isoproterenol.

In summary, from the molecular analysis of rat RP4 gene expression, we have identified a novel salivary-specific CRE that is crucial for cAMP-induced gene expression in salivary glands. The lack of cAMP responsiveness of the salivary promoter in HeLa cells and the lack of sequence-specific DNA binding of HeLa nuclear proteins to the RP4 promoter point to the existence of a SCBP that is somehow critical for cAMP responsiveness in salivary cells. Re-

```
                    CONSENSUS CRE: TGACGTCA
RP 4 (-136) CTGGGCAAATGTCCCAGTGTGGAGTCAG (-109)
RP13 (-136) ----------A---------------- (-109)
RP15 (-133) ---------A---------------A-A (-106)
MP 2 ( -88) A--------A---------CAC-CAA-- ( -61)
MP 6 (-128) --A---T--A------AA-AA------- (-101)
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Figure 8. Sequence comparison of 5′ regions among PRPs from rat and mouse. The corresponding 5′-flanking regions of the PRPs from rat RP4, 13, and 15 (Lin and Ann, 1991), mouse MP2 (Ann and Carlson, 1985), and MP6 (Roberts et al., 1991), that share the greatest homology to the sequence from −136 to −109 of the rat RP4 gene, and consensus CRE (Roesler et al., 1988; boldface) are aligned. The dashes indicate identical bases and differences are as shown.

cently, a cDNA that encodes a novel nuclear protein (SCBP) was cloned by screening salivary lambda-gt11 expression library with the identified salivary-specific CRE (H. H. Lin and D. K. Ann, unpublished data). Characterization of the cDNA will allow us to examine the cell type specificity of this SCBP factor, and forced expression of this cDNA in HeLa and a variety of cell types should help to determine whether this factor has broad significance in salivary-specific gene expression.

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