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Coordination of murine parotid secretory protein and salivary amylase expression

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PSP, parotid secretory protein, and salivary amylase are the major secretory proteins of mouse parotid gland where they appear in a constant ratio. Here we describe the isolation of the PSP gene and show through expression analysis on this and the salivary amylase gene that the two genes are transcribed in a coordinate fashion in adult animals, whereas the activation profiles are different during postnatal development. An explanation is put forward that involves activation of the genes at different stages of the acinar cell differentiation, leading in adults to the maximal and thus proportionate expression.

Key words: expression coordination/mouse PSP gene/parotid gland amylase/postnatal development/promoter sequence

Introduction

Gene activity is often regulated at the transcription level. Expression analysis of deletion series of cloned genes shows that the main determinants for specific expression are localized in the gene promoter, typically within the first 300 nucleotides upstream of the transcription start (Brinster *et al.*, 1982; Stuart *et al.*, 1984; Ornitz *et al.*, 1985) but determinants can also be found further upstream as well as downstream (*loc. cit.* and Banerji *et al.*, 1983; Kosche *et al.*, 1985). In an array of cases the induction or tissue specific coordination of gene activity seems to be realized through common short transcription signals in the promoters, often palindromic, that function as binding sites for regulator proteins. The homeostatic regulation by glucocorticoid hormones of a number of genes is mediated through a short promoter sequence via the binding of a receptor protein (Rousseau, 1984; von der Ahe *et al.*, 1985). Five serine proteases, expressed in rat exocrine pancreas, have a short conserved sequence in the respective promoters that may relate to their common tissue specific expression (Walker *et al.*, 1983; Swift *et al.*, 1984). Similarly, the induction by heavy metal ions of metallothionein genes in mouse and man is mediated through small promoter signals (Karin *et al.*, 1984), and it has been documented for both a chicken β-globin gene and *Drosophila melanogaster* heatshock genes that a regulatory protein binds to a specific short promoter sequence (Emerson *et al.*, 1985; Parker and Topol, 1984; Wu, 1985) that in the latter case is known to induce gene activity (Pelham, 1982). This kind of promoter response may turn out to be the general mechanism in eukaryotes to regulate and coordinate gene activity particularly for highly expressed proteins. Alternative regulatory mechanisms involving steps other than transcription are found but they may especially relate to genes for housekeeping proteins (Carneiro and Schibler, 1984).

The two main secretory proteins of mouse parotid glands, salivary amylase and PSP (partotid secretory protein), are coordinated both in their tissue specific expression and in their relative levels in saliva (Owerbach and Hjorth, 1980). The parallel levels in saliva are likely to be regulated during transcription because the levels of translatable mRNA are coordinated (Madsen and Hjorth, 1985). We seek the regulatory mechanisms responsible for these two kinds of coordination. They will apparently involve genetically transacting elements, because the two genes of concern are unlinked, and may therefore rely on the kind of short transcription signals mentioned above. This situation provides special opportunities to acquire new information about the regulation of gene expression.

Salivary amylase is a common secretory enzyme in salivary glands of many mammals (Karn and Malacinski, 1978). In the mouse it is determined within a large gene complex containing, in addition, several genes for pancreatic amylase (Nielsen, 1982). Only one gene is present for salivary amylase which is mainly expressed in the parotid glands (Hjorth, 1982; Schibler *et al.*, 1982). Low transcription of the gene, however, also takes place in both liver and pancreas but from a different promoter (Hagenbüchle *et al.*, 1985). PSP is very abundant in mouse parotid glands, and we have detected PSP mRNA in parotids of rat, white-necked fieldmouse, and bank vole. In man, the PSP mRNA is present in both parotid and submandibular glands. Recently, we determined the major part of the mouse PSP mRNA sequence from isolated cDNA clones (Madsen and Hjorth, 1985). These clones were used to isolate recombinant λ-phages containing fragments of the murine PSP gene. Here we report the structure and the promoter sequence of the gene and compare it and its expression with that of salivary amylase. We show that the mRNA levels for the two proteins are coordinated in fully differentiated parotid glands, but that the increase in levels is disproportionate during the ontogenic development. A model for the regulation of expression patterns is discussed.

Results

Cloning, isolation and characterization of the PSP gene

The PSP gene was isolated from a λ-phage library of mouse strain C3H.*Amy*^{YBR} spleen DNA using the vector λL47.1. Six out of seven positive plaques, λYP1-7, were purified from 1.2×10^6 plaques of the nonamplified library, while one, λYP2, was rejected due to its low titer. As probe for this screening we used a ^{32}P-nick-translated PSP cDNA clone, pMPd39, which covers the 3′ part of the mRNA (Madsen and Hjorth, 1985). In further analysis we also used the clone pMPd12 which covers the 5′ part of the mRNA up to the initiation methionine as shown in Figure 1A. Restriction endonuclease sites were determined by complete digestion of DNA from the six individual λYPs with the enzymes *Eco*RI, *Hin*dIII, *Bam*HI, *Sal*I and *Xho*I, and in addition of λYP1 DNA with *Xba*I (Figure 1B). The sequence of restriction sites were furthermore determined from partial digestions of the λYP DNA with *Bam*HI, *Eco*RI and *Hin*dIII, by Southern blot analysis using as probe one of the two *Bam*HI/*Hin*dIII fragments of λL47.1 which flank the insert (Mikkelsen *et al.*, 1985). Restric-

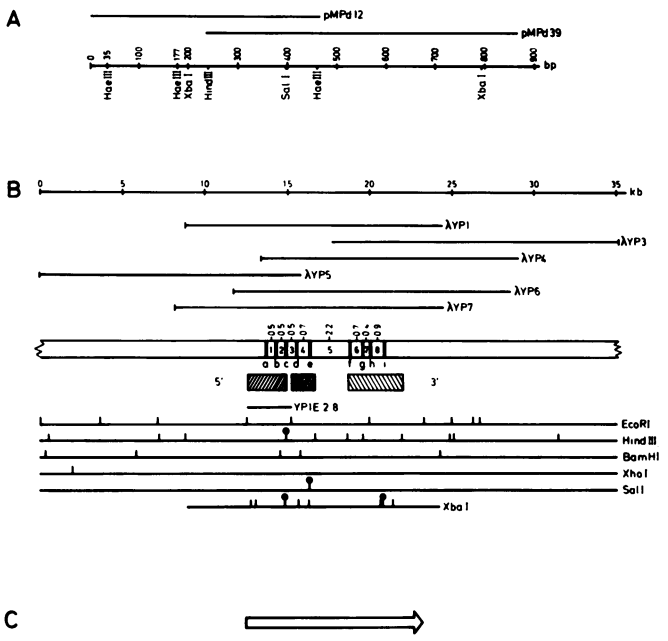

Fig. 1. Structure of the PSP gene. (**A**) Restriction map of two overlapping PSP cDNA clones with 5′ end to the left (Madsen and Hjorth, 1985). (**B**) Combined restriction map of recombinant λ-phages with the PSP gene. Terminal bar indicates left arm of the individual phages. Restriction fragments from *Eco*RI/*Hin*dIII double digests hybridizing to pMPd12 and pMPd39 are shown as ▨ and ▧ respectively, and restriction sites in exons by a black 'knot'. Shown on the middle bar is the exon distribution derived from heteroduplex analysis. Exons are labelled a−i. Lengths of introns 1−8 are given in kb. (**C**) Extent and direction of transcription as derived from hybridization to phage *Eco*RI restriction fragments of *in vitro* elongated transcripts from parotid gland nuclei.

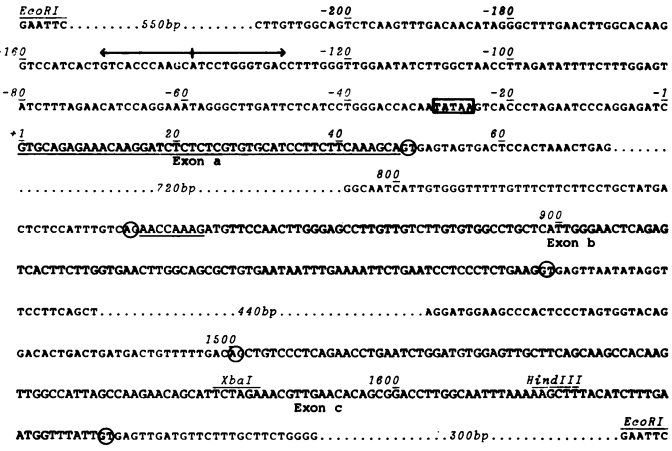

Fig. 2. Nucleotide sequence of the 5′ end of the PSP gene. Parts of the sequence of YP1E2.8 are given, using boldface letters for exons. The deduced GT and AG splice points are circled, whereas the sequence derived by primer extension of PSP mRNA is underlined. Transcription starts at +1 and the two arrows upstream of this indicate an inverted repeat. A printing error was detected in the published cDNA sequence (Madsen and Hjorth, 1985) corresponding to G No. 871.

tion fragments containing exons were identified by hybridization with pMPd12 and pMPd39 and the orientation of the gene was thereby deduced. The inserts of the six recombinants overlap and span a total of 35 kb, suggesting the presence of just one PSP gene per haploid genome.

By Southern blot technique, restriction analysis of C3H.*Amy*^{YBR} genomic DNA confirms the localization in the gene of the sites for *Bam*HI, *Eco*RI and *Hin*dIII, as derived from the λ-cloned DNA and reveals no additional bands (data not shown) suggesting that all genomic PSP sequences were cloned. A second PSP allele in the strain DBA (Owerbach and Hjorth, 1980) revealed two new *Eco*RI sites closer to the gene on each side. There were no signs of the alternative *Eco*RI restriction fragments in either strains which would have indicated multiple genes. This agrees with both the consistent single restriction map derived from the overlapping λ-clones and the earlier conclusion that *Psp* is a single gene, as inferred from the alternative protein structures (*loc. cit.*).

Size and location of the exons were determined by analysis of heteroduplex formation between DNA from λYP6 and the *Eco*RI linearized cDNA clones pMPd12 and pMPd39 as well as parotid gland poly(A)$^+$ RNA from mouse strain C3H.*Amy*^{YBR}. A total of nine exons and eight introns were found and their sizes were estimated from electron micrographs (Christiansen and Christiansen, 1983). The leader exon was detected only by using the poly(A)$^+$ RNA suggesting that the PSP mRNA has a 5′ part that is not contained within the cDNA clones used. All exons are small, i.e. at the level of variance of measurement, 0.1−0.2 kb, while intron sizes span form 0.4 to 2.2 kb (Figure 1B). The derived exon map of the PSP gene was unambiguously aligned with the restriction map by using both the fact that pMPd12 and pMPd39 partially overlap, and that only one or few restriction sites for *Hin*dIII, *Sal*I and *Xba*I occur both in the PSP cDNAs and in the λYP inserts.

The extension of transcription from the PSP gene was determined by hybridizing ^{32}P-labelled *in vitro* elongated nascent transcripts from isolated parotid gland nuclei (see next paragraph) to Southern blots of *Eco*RI restriction fragments of λYP1, λYP3 and λYP5 DNA (data not shown). The transcription starts no further than 0.8 kb upstream of exon a and stops within 2.4 kb downstream from exon i (Figure 1C) demonstrating the entire PSP gene transcription unit is contained within the DNA stretch cloned in the λYPs.

The precise transcription start was localized by identifying the PSP mRNA leader sequence, derived by primer extension, in the sequence of a 2.8 kb *Eco*RI restriction fragment from λYP1. We used the 142 bp *Hae*III fragment from pMPd12 to prime the cDNA synthesis of poly(A)$^+$ RNA from parotid glands of mouse strain C3H.*Amy*^{YBR}. The synthesis was performed in the presence of chain terminating dideoxynucleotides and [^{32}P]dATP, and the products analysed on squencing gels. The obtained sequence beyond that known from pMPd12 constitutes 56 nucleotides and is underlined in Figure 2 which presents parts of the sequence of YP1E2.8 (Figure 1B). Omitting dideoxynucleotides from the reaction mixture resulted in the synthesis of one major and three shorter, very minor products. We interpret the minor products to be the result of premature terminations of the reverse transcription and conclude that only one cap site is used in the PSP gene of parotid gland cells. This defines a likely cap site at G No. +1 and a TATA box around base No. −28. By comparing the nucleotide sequence of YP1E2.8 to the PSP cDNA sequence and applying the GT−AG splice rule, a detailed 5′ gene structure is derived (Figure 2) that is in keeping with that previously found (Figure 1B). Exon a contains 47 nucleotides of the mRNA leader. Eight hundred and one nucleotides downstream starts exon b coding the last eight nucleotides of the leader, the 20 amino acid signal peptide, and the first 20 residues of the native protein. Five hundred and twenty-four nucleotides further down is exon c, coding 47 amino acids.

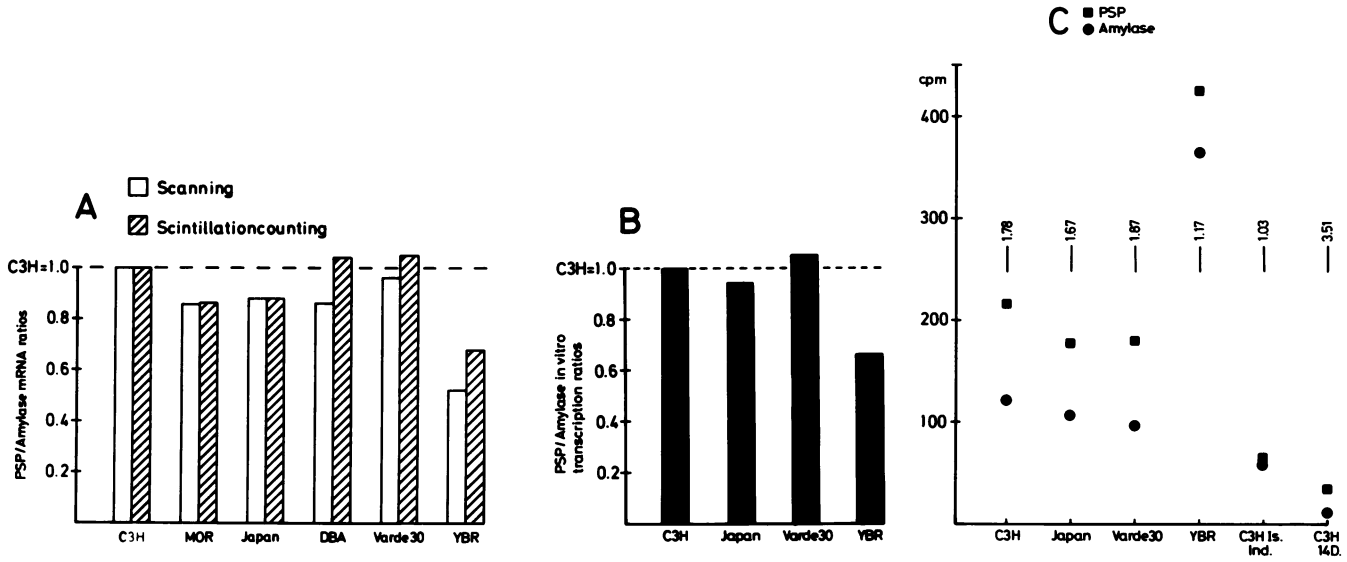

Fig. 3. Coordination of PSP and salivary amylase expression in different mouse strains. (**A**) Ratios of steady-state PSP and amylase mRNAs from parotid gland. The data were obtained from Northern blots hybridized with ^{32}P-labelled pMPd39 and pMSa104, by scanning (open columns) or scintillation counting (hatched columns) of excised hybridizing bands. In each case the columns are normalized to C3H = 1.0, and represent the mean values from three or four determinations. (**B**) Ratios of *in vitro* elongated PSP and amylase RNAs transcribed in isolated parotid gland nuclei. Ratios are calculated from results presented in **C**, normalized to C3H PSP/amylase = 1.0. (**C**) Results of *in vitro* transcription experiments in isolated parotid gland nuclei. Purified ^{32}P-labelled RNA was hybridized to nitrocellulose membranes with 4 μg dots of pUC13, Amy: pMSa104 (Schibler *et al.*, 1980) and PSP: pMPd1239, a pSP64 clone (Melton *et al.*, 1984) containing the combined cDNA of pMPd12 and pMPd39. Dots were excised and counted in a liquid scintillation counter, and results adjusted by subtracting the pUC13 dot counts as background. Vertical figures indicate the PSP/amylase *in vitro* transcription ratios calculated from the results. The results quoted represent one experimental determination for each mouse line except in the case of the 14-day-old mice (C3H 14D) where the results are mean values from three independent experiments, as counts from the Amy and PSP dots in these experiments were only about three times the background level. For information bearing on isoproterenol induction experiments (C3H Is.Ind.) see text and Figure 4.

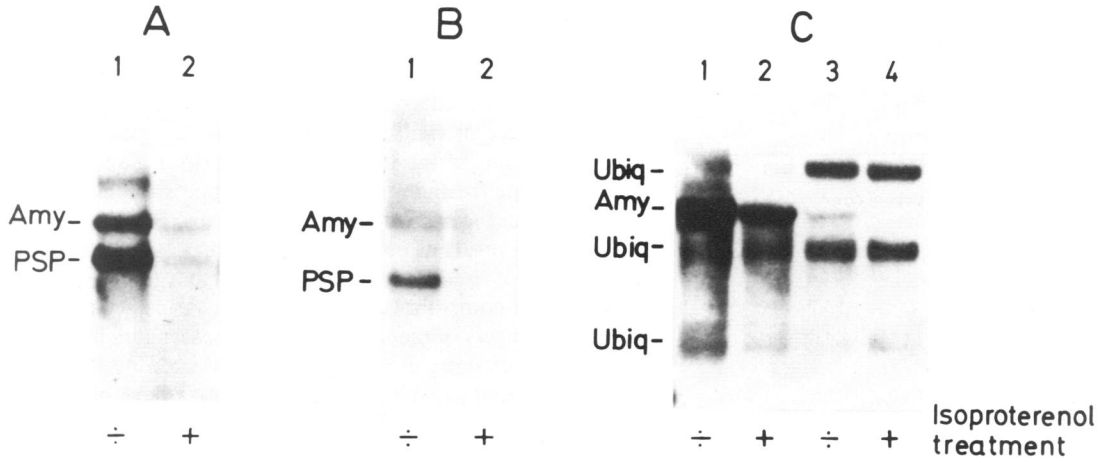

Fig. 4. Effects of isoproterenol treatment on the expression of the PSP and salivary amylase genes in strain C3H. Mice treated (+) received daily injections for 1 week with 0.2 mg isoproterenol per animal. (**A** and **B**) Northern blots of 20 μg RNA from parotid gland (**A**) and submaxillary gland (**B**) hybridized to both ^{32}P-labelled pMPd39 and pMSa104. (**C**) Northern blot as **A** (**lanes 1** and **2**) and **B** (**lanes 3** and **4**) hybridized to both ^{32}P-labelled pMSa104 and *Xho*I fragments of λHUb13 coding for human ubquitin (Wiborg *et al.*, 1985).

Expression of the PSP and salivary amylase genes

Northern blot analysis of RNA from various mouse tissues shows that PSP mRNA predominates in the parotid glands. It is present in considerably smaller amounts in the submaxillary glands (Figure 4) and the sublingual glands, and in even lower amounts in pancreas but is undetectable by us in the liver (data not shown).

An earlier observation (Owerbach and Hjorth, 1980) showed a constant ratio in the protein levels of PSP and salivary amylase in mouse strains with different amylase gene complexes. Only one strain, YBR, diverged from this pattern, having about two-fold higher amylase production (Hjorth, 1979). To establish at what level this apparent coordination in expression is regulated, we have observed steady-state ratios of the two messengers in six congenic mouse strain with C3H/As genetic background, differing only in the amylase complexes and having the *Psp*^{b} allele, except for the DBA strain that has *Psp*^{a} and is not congenic to C3H/As. The PSP:amylase RNA ratios were determined from Northern blots of total parotid gland RNA by hybridization to PSP and amylase cDNA probes (Figure 3A). The results obtained are in agreement with the previous observations of the protein levels. Five strains show, within the limits of experimental variation, a constant ratio of the two messengers, while YBR has a lesser value for the ratio, i.e. relatively more amylase than PSP. This same pattern was also obtained in experiments with *in vitro*

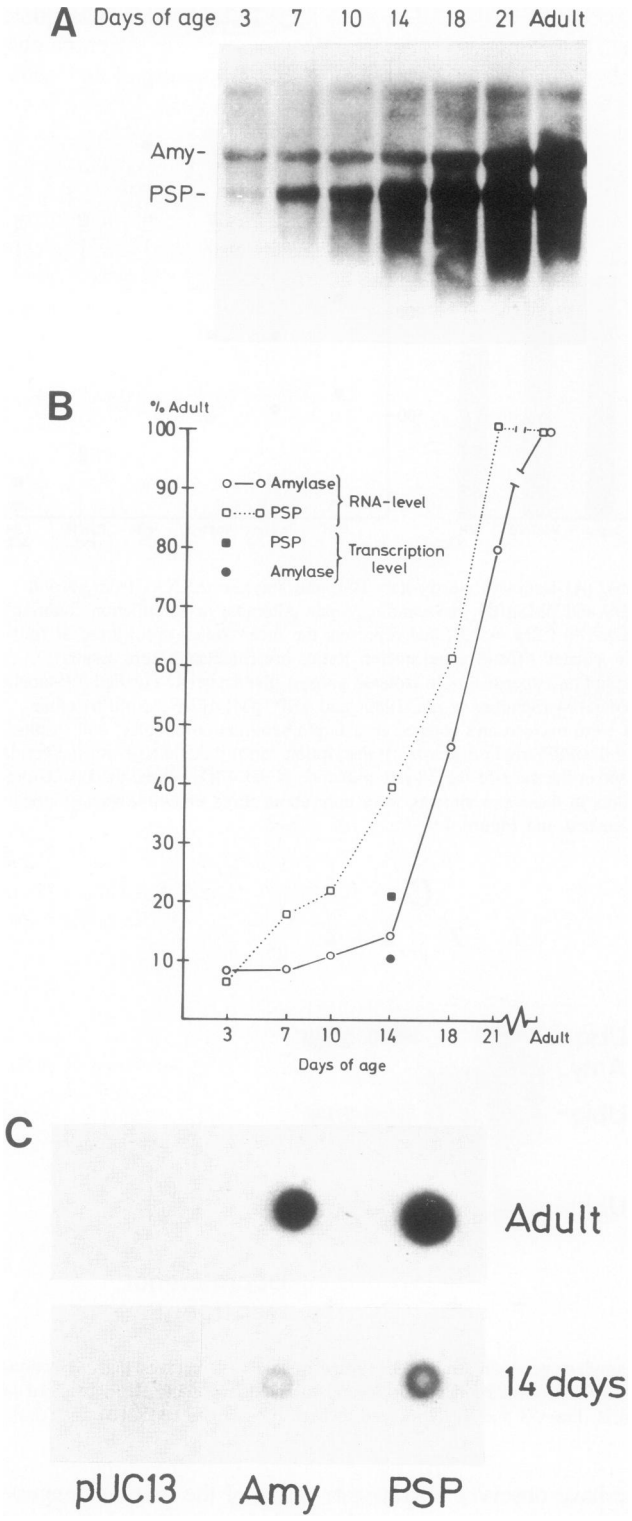

Fig. 5. Ontogeny of the PSP and salivary amylase gene expression in parotid glands of strain C3H. (**A**) Northern blot of 20 μg RNA from mice of various ages hybridized to ^{32}P-labelled pMPd39 and pMSa104. (**B**) Graph showing mRNA levels as determined from scintillation counting of bands in **A**. Adult level equals 100%. Filled symbols represent 14 days relative to adult transcription derived from scintillation counts of run-on RNA selectively bound to dots of Amy and PSP shown in **C**. (**C**) *In vitro* synthesized ^{32}P-labelled RNA from comparable run-on assays with nuclei from adult and 14-day-old mice hybridized to nitrocellulose filters with 4 μg plasmid DNA dots as described in Figure 3C, and exposed together for the same length of time.

elongated nascent transcripts from isolated parotid gland nuclei (run-on transcription). The purified [^{32}P]UTP labelled RNA from assays with standard number of nuclei and radioactivity was used in hybridizations to dots of excess amounts of specific DNAs fixed on nitrocellulose membranes. After hybridization, the RNA selectively bound to PSP and amylase cDNA was measured and the ratio normalized to C3H (Figure 3B). These are in concordance with those obtained for the steady-state mRNA ratios (Figure 3A). Thus, the coordination of PSP and amylase expression seems to depend solely on their mutually correlated level of gene transcription. The absolute counts for selected PSP and amylase run-on RNA were quite similar for the strains C3H, Japan, and Varde 30. Thus the reproducibility of the assay indicates that it measures the transcription level of the genes directly. The nearly double transcription for both genes in strain YBR is therefore surprising and at present under further analysis. The molar ratio in transcription rate between PSP and amylase genes are 3.5 and 2.3 for strains C3H and YBR, respectively, since the selected counts in run-on RNA (Figure 3C) has to be corrected by the factor 1659/867 resulting from the length of the employed cDNAs.

To test this coordination further, we performed a series of experiments where mice were subjected to physiological stress. Neither starvation for 24 h, nor starvation followed by feeding for 1 h introduces any changes in the ratio between the two messengers or in their abundance in relation to total RNA (data not shown). In contrast, dramatic changes were observed when animals were treated with a saliva-stimulating agent, isoproterenol, which on continued treatment stimulates growth of gland cells, mainly through cell expansion (Seifert, 1967). After this treatment, the parotid glands synthesize a vast amount of proline rich proteins (Mehansho *et al.*, 1985). Daily injections for a week with 0.2 mg isoproterenol per animal, made parotid and submaxillary glands assume approximately four times normal weight. This short treatment will cause only insignificant alterations in the number of the various gland cell types. However, the treatment significantly suppressed the mRNA levels of PSP and amylase (Figure 4A and B). To see whether this effect is a result of generally altered processing or breakdown kinetics, we used a ubiquitin probe (Wiborg *et al.*, 1985) as standard in a comparative Northern blot. As is seen from Figure 4C, the levels of the three ubiquitin messengers in relation to total RNA amounts, are essentially unaffected by the isoproterenol treatment in both parotid and submaxillary glands whereas those of amylase (Figure 4A, B and C) and PSP mRNAs (Figure 4A and B) are significantly lowered. Thus, the reduction in PSP and amylase mRNA appears to be specific.

In vitro transcription using parotid gland nuclei from isoproterenol-treated mice shows that at least the major part of this effect is performed through an altered transcripton rate. The selected counts of RNA from run-on transcription are considerably reduced for both the PSP and amylase genes (Figure 3C). In this extreme stress situation the ratio of PSP to amylase transcription is lowered to 1.0 from the value of 1.8 (not corrected for cDNA length) in untreated mice, resulting from a more pronounced reduction in the PSP gene transcription.

A possible coordination during the developmental activation of the two genes in question was investigated through Northern blots of parotid gland RNA isolated from young mice at day 3, 7, 10, 14, 18 and 21 after birth. Both genes are expressed at low levels at the youngest state investigated, i.e. 3 days, but whereas PSP-messenger content increases gradually with age from this time, that of amylase increases more abruptly between

14 and 18 days of age (Figure 5A, B and C). The ubiquitin mRNAs stay the same within a factor of 2 (data not shown).

The observed ontogenic activation of PSP and amylase expression seems dependent on transcription. Nuclei from 14-day-old C3H mice give very reduced run-on transcription relative to adult mice of the same strain (filled symbols in Figure 5B) and the values are in fairly close agreement with the respective values for the steady-state mRNA levels. Moreover, the ratio between transcription of PSP and amylase genes in 14-day-old mice, 3.5, is the highest value observed (Figure 3C), showing a preference for the PSP gene that is in concordance with the mRNA levels (Figure 3A). These results, taken together, are clearly compatible with a situation where the ontogenic progression in expression is solely transcriptionally caused.

Discussion

PSP and salivary amylase are the major proteins in mouse saliva and they appear in the same constant ratio in all mouse strains examined (except for YBR) although the genes are unlinked (Owerbach and Hjorth, 1980). Earlier we have described the isolation, structural analysis, and sequence of PSP cDNA clones (Madsen and Hjorth, 1985) and have found the same translation ratio for the messengers of PSP and salivary amylase in a heterologous *in vitro* system compared to *in vivo*, indicating that a possible coordinate expression of the two genes is regulated through the level of mRNA.

Here we report the isolation and structural characterization of genomic PSP clones which show beyond any reasonable doubt that *Psp* is a single copy gene. In parotid gands of adult animals we find for the two genes *Amy-1* and *Psp* a constant ratio both between the steady state mRNAs and between the *in vitro* elongated transcripts. Normal physiological variation does not alter this situation and when the glands are subjected to severe stress by treating the mice with isoproterenol, the *in vitro* transcription from the two genes, though forced somewhat out of the otherwise observed constant ratio, follows the same major tendency.

The developmental activation of the genes, on the contrary, is not concomitant. These observations are complicated by the fact that the salivary amylase gene is transcribed from two promoters in parotid glands, designated the liver and parotid promoters (Schibler *et al.*, 1983). The proximal and weaker liver promoter is activated before birth and remains active at almost the same level throughout life. In contrast, the parotid promoter becomes active only from about 14 days of age, causes increasing expression throughout the youth of the mouse and accounts for about 97% of the *Amy-1* transcription in adults (Shaw *et al.*, 1985; Schibler *et al.*, 1983; Hagenbüchle *et al.*, 1985). Therefore, the low levels of amylase mRNA observed at the earliest developmental stages are a result of transcription from the weak promoter, whereas the dramatic increase around 14 days of age (Figure 5) must be attributed to the activation of the strong parotid promoter. The activation of the strong promoter is thus distinguishable from that of PSP in being delayed by about 10 days and, once activated, the increase is more abrupt. This latter observation could be connected with the more differentiated state of the gland when the strong amylase promoter is activated. In this respect it is interesting that the activation of the strong promoter of the salivary amylase gene has been shown to be an off−on event and the observed gradual increase during development is the result of progressive cellular commitment (Shaw *et al.*, 1985). It seems likely that the activation of the PSP promoter, though occurring earlier, is also dependent on the differentiation of the cell in a similar off−on fashion. Considering this, the observations on the ontogeny might, in effect, be observations of the differentiation of the committed gland cells in respect to activation of the investigated genes. Results from *in vitro* transcription experiments compared to mRNA levels indicate that the activation of both genes in question is transcriptionally based (Figure 5B). This is also the case with the observed coordination in adult animals (Figure 3A and B), and therefore the explanations for the expression patterns in various states/conditions must be sought among mechanisms regulating transcription.

Most likely both genes are subjected to transcriptional regulation by several factors exerting their effect by interacting on promoters. In a situation where some of these factors are different for the two genes, there is no *a priori* reason to expect a parallel developmental activation, and neither was this observed. The explanation for the constant transcription ratios of the two genes at the adult stage could then be that both are fully activated so that their products appear at constant ratio through all steps of the expression process. All our data can be accounted for by these explanations.

In addition, the genes may have some common tissue specific transcription factor(s). Therefore we searched for a putative common factor target sequence in the 5′ upstream region of the PSP gene and the 181 bp parotid promoter of *Amy-1*^{a} (Young *et al.*, 1981). No overall similarity was apparent but a short, fairly conserved 24 bp element was found around −130 bp of the two genes with 60% homology around a central CAT motif. The PSP version of the element has a pronounced palindromic structure (see Figure 2) and its sequence is different from known regulatory DNA sequences such as those referred to in the introduction. Future expression studies with the promoters will verify whether this element plays a significant role in the tissue specific expression or the coordination of *Psp* and *Amy-1*.

Materials and methods

Mouse strains

We have used the short names C3H, YBR, Varde 30, MOR, and Japan for the following congenic strains: C3H/As, C3H.*Amy*^{YBR}, C3H.*Amy*^{w9} (Nielsen, 1982), C3H.*Amy*^{MOR} (Hjorth *et al.*, 1980). The Japan strain was recently bred congenic to C3H/As, using a mouse with electrophoretic amylase phenotype salivary B, pancreatic A kindly provided by Dr K.Moriwaki, Mishima, Japan. DBA/2 Bom was purchased from Gl. Bomholtgaard, Ry, DK-8680.

Construction and analysis of genomic clones

Recombinant λ-phages were constructed from 15−20 kb fragments of partial *Sau*3A digested spleen DNA from mouse strain C3H.*Amy*^{YBR} using λL47.1 (Loenen and Brammar, 1980) as *Bam*HI substitution vector (Mikkelsen *et al.*, 1985). Phages packed *in vitro* were plated on *Escherichia coli* strain K802 and positive plaques identified by *in situ* hybridization using the PSP cDNA pMPd39 as probe. Post hybridization washes were at 60°C in 1 × SSC. Positive plaques were purified by replating on *E. coli* strain ED8910 and DNA isolated from 20 ml phage cultures (*loc. cit.*).

Northern analysis

RNA was purified by the guanidinium thiocyanate/caesium chloride method (Maniatis *et al.*, 1982). The RNA, 20 μg per lane, was electrophoresed in 1.4% agarose gels containing 2.2 M formaldehyde and 1 × MOPS buffer, then blotted onto Gene Screen membranes according to the manufacturer's instructions. For quantification of specific mRNAs after hybridization, radioactive bands were excised and counted 20−100 min in a Beckman HP-250 liquid scintillation counter. Counting of areas from hybridized filters outside the RNA lanes gave unreproducible values, and therefore countings of unhybridized membranes were used for background subtraction.

Primer extension of PSP mRNA

The 142 bp *Hae*III fragment of pMPd12 (Figure 1A) was used as primer. The electrophoretically purified fragment (0.25 μg) along with 1 μg parotid gland poly(A)$^+$ RNA from mouse strain C3H.*Amy*^{YBR} were denatured by heating to 73°C for 10 min in 80% formamide solution (Craik *et al.*, 1984) including 0.5 U/μl RNasin, and left at 52°C for 2 h to anneal. The RNA/DNA hybrids

were precipitated by ethanol, dissolved in 15 μl RT-buffer (40 mM Tris−HCl pH 8.3, 10 mM KCl, 6 mM $MgCl_2$, 1 mM DTT) and stored at −20°C. Of this mixture 0.5 μl was used for each sequencing reaction which was set up as described by Zimmern and Kaesberg (1978) with the following modifications. The reactions were performed in 4 μl RT-buffer containing 10 μCi [^{32}P]dATP (3000 Ci/mmol) and 3 U AMV reverse transcriptase. The nucleotide concentrations were 5 μM in dATP and 300 μM in the other three dNTPs, except for the one also present in the dideoxy form, which was at 50 μM. The ddNTP concentration was 5 μM in the ddC, ddG and ddT reactions, and 2.5 μM in the ddA reaction. Reactions were carried out for 20 min at 50°C and chased for another 20 min at 50°C by adding 1 μl of a 0.5 mM solution of the four deoxynucleotides. The products were analysed by electrophoresis on 6% sequencing gels and subsequent autoradiography.

Elongation of nascent RNA chains in isolated parotid gland nuclei

The parotid gland nuclei were purified by modification of the method described by Schibler *et al.* (1983). The parotid gland tissue was minced thoroughly on ice in a Teflon/glass 0.1-mm clearance homogenizer with 25 ml per g tissue of 0.3 M sucrose in buffer A (60 mM KCl, 15 mM NaCl, 0.15 mM spermin, 0.5 mM spermidine, 14 mM beta-mercaptoethanol, 0.5 mM EGTA, 2 mM EDTA, 15 mM HEPES pH 7.5). After filtering through 40-μm nylon gauze, the homogenate was layered over 10 ml 30% sucrose in buffer A, and centrifuged for 15 min at 3000 r.p.m. at 4°C in a Sorvall HB4 rotor (swing out). The nuclei pellet was resuspended in 25 ml/g tissue of 0.3 M sucrose in buffer A, and treated with 10 μg/ml RNase A for 30 min at 0°C. The nuclei were sedimented through 10 ml 30% sucrose in buffer A three times, resuspending in 25 ml/g tissue of 0.3 M sucrose in buffer A in between, and adding 0.5 mg/ml yeast RNA in the last resuspension. The final nuclei pellet was resuspended in 1 ml 30% sucrose in buffer A, transferred to siliconized micro-tubes, and centrifuged for 30 s at 4°C. The pellet was resuspended in nuclei storage buffer (20 mM Tris−HCl pH 7.9, 75 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT, 0.125 mM PMSF, 50% v/v glycerol, 0.1 U/μl RNasin) at a concentration of approximately 10^5 nuclei per μl and stored at −80°C. The yield varied between 0.5 and 2 × 10^8 nuclei/g tissue.

The *in vitro* elongation was carried out in 250-μl reaction mixture containing 100 mM Tris−HCl pH 7.9, 50 mM NaCl, 0.4 mM EDTA, 0.1 mM PMSF, 1.2 mM DTT, 350 mM $(NH_4)_2SO_4$, 4 mM $MnCl_2$, 29% v/v glycerol, 10 mM creatine phosphate, 130 μg/ml creatine phosphokinase, 1 mg/ml heparin sulphate, 1 mM each of GTP, ATP and CTP, 2 μm UTP, 1 μCi/μl [^{32}P]UTP (3000 Ci/mmol), 40 U/ml RNasin and 4 × 10^7 nuclei/ml. After incubation at 26°C for 15 min the transcription reaction was stopped by adjusting the solution to 6 mM $MgCl_2$, 35 μg/ml DNase I, 90 μg/ml yeast RNA and incubating for 5 min at 26°C. The mixture was deproteinized for 30 min at 37°C in 1% SDS, 4.75 mM EDTA, 10 mM Tris−HCl pH 7.5, and 90 μg/ml Proteinase K. After phenol and chloroform extractions, the nucleic acids were ethanol precipitated. The pellet was resuspended in 500 μl 20 mM HEPES pH 7.5, 5 mM $MgCl_2$, 1 mM $CaCl_2$ and DNase digestion repeated by adding 25 μg DNase I and incubating at 37°C for 30 min. EDTA was then added to 15 mM, SDS to 1%, proteinase K to 50 μg/ml and the incubation was continued for another 30 min. After phenol and chloroform extractions, the free triphosphates were removed from the RNA by five sequential ethanol precipitations with 2 M NH_4Ac. The RNA was resuspended in H_2O, heated at 65°C for 5 min and used for hybridizations.

Hybridizations

When probing phages fixed on nitrocellulose filters or Southern blots of digested phage DNA with PSP cDNA, we used [^{32}P]dATP that was incorporated by nick-translation as label. The prehybridization (for 4 h) and the hybridization (for 12 h) were carried out at 65°C in 4 × SSC, 0.2% Ficoll, 0.2% PVP, 0.1% SDS 50 μg/ml sheared, denatured, salmon sperm DNA.

Applying RNA from *in vitro* elongated transcriptions as probe to Southern blots or dot blots, prehybridization (for 12 h) and hybridization (for 48 h) were performed in 50% deionized formamide, 5 × SSC, 0.02% BSA, 0.02% Ficoll, 0.02% PVP, 1% SDS, 10 μg/ml tRNA, at 42°C, including 200 μg/ml sheared, denatured, mouse DNA in the Southern blot hybridizations to compete out hybridization of repetitive sequences (Schibler *et al.*, 1983).

Sequencing procedure

The 2.8 kb *Eco*RI fragment of λYP1, YP1E2.8 was cloned into the *Eco*RI site of M13mp18 in both orientations. A series of progressive deletions was produced by varying the time length of nuclease *Exo*III digestion of *Pst*I/*Sma*I opened RF DNA (Yanish-Perron *et al.*, 1985). For removal of the resulting single stranded ends we used S1 nuclease instead of *Exo*VII.

Sequence of individual clones were determined by the dideoxynucleotide chain termination method Sanger *et al.* (1977) modified by Biggin *et al.* (1983), using the 15-mer primer from Biolabs and [α-^{32}P]dATP. Sequencing data were assembled with the aid of an Apple IIe computer, using programs developed by Larson and Messing (1982).

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