Involvement of Nuclear Orphan Receptor NGFI-B in Transcriptional Activation of Salivary-specific R15 Gene by cAMP*

(Received for publication, May 2, 1996, and in revised form, August 20, 1996)

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Proline-rich proteins (PRPs) are selectively expressed in the acinar cells of the salivary glands and are inducible by β-agonist isoproterenol and dietary tannins. In the previous studies of rat PRP gene, R15, the 5′-flanking region up to −1.7 kilobase pairs (kb), which was thought to contain the necessary proximal regulatory elements, failed to confer the catecholamine isoproterenol and dietary tannin inducibility to the transgene expression in the salivary glands of transgenic mice. Here we analyzed distal 5′-flanking region of R15 in order to understand the mechanisms of tissue-specific and inducible gene regulation. An upstream regulatory region located between −2.4 and −1.7 kb of the R15 5′-flanking region is demonstrated to be indispensable for the salivary-specific and inducible reporter gene expression in vitro, by transgenic approach. Element(s) within the 0.7-kb (−2.4 to −1.7) region that is able to cis-activate the expression of a heterologous reporter gene expression is further elucidated by transient transfection assays in vitro. Three distinct nuclear orphan receptor NGFI-B regulatory sequences are identified within a 184-base pair (bp) minimal control region extended from −1985 to −1812 nucleotides relative to the transcription start site. When reporter gene containing this 184-bp control region and heterologous promoter was cotransfected with the NGFI-B expression construct, a transactivation that mimics the effect of CAMP is observed in the parotid cells. Finally, mutations on all three identified NGFI-B binding sites and coexpression of a dominant negative mutant construct, pCMV-NGFI-B(Δ25–195), abolish this transactivation mediated by NGFI-B. In summary, these data suggest that the inducible nuclear orphan receptor NGFI-B may participate in the regulation of salivary acinar cell-specific and inducible expression of the rat R15 gene via three distinct distal NGFI-B sites.

The salivary proline-rich proteins (PRPs)* have been demonstrated to function by forming a protective coat on the hard and soft tissues of the mouth. In rodents, these PRPs are exclusively synthesized and secreted by the salivary acinar cells, and PRP expression is dramatically induced by β-adrenergic stimulation or by ingesting dietary tannins (1–3). The response to dietary tannins may suggest that one of the functions of PRP is to protect organisms from the toxic effects of tannins, common in the human diet (3). Furthermore, the expression of PRP in rodent parotid glands is developmentally regulated in parallel with the postnatal differentiation of salivary acinar cells (4, 5). Initially, PRP transcripts are barely detected in parotid glands of the postnatal day 14 mice by in situ hybridization and only in a few well-differentiated acinar cells (6). Then there is a burst of PRP transcription that coincides with the appearance of high density of functional β-adrenergic receptor starting at postnatal day 21. But toward the end of the weaning period, only low levels of PRP expression are detected in parotid acinar cells (6). After postnatal day 28, the rodent PRP protein and mRNA levels increase as a consequence of the experimental conditions such as isoproterenol (Ipr) injection and tannin feeding that raise the intracellular cAMP levels, suggesting that the tissue-specific induction of PRP expression is regulated at the level of transcription (1, 2, 7–10).

While the components of the signal transduction pathways are mostly of ubiquitous nature, cAMP induction of a particular gene is often restricted to specific cell types and tissues. We have chosen the regulation of PRP gene expression as a model system to investigate the basis for tissue specificity of cAMP induction. Sequence analyses of PRP genomic clones and in vitro transient transfection assays indicate the presence of salivary-specific cAMP-responsive elements in the proximal 5′-flanking region of the PRP genes (11–14). In addition, nuclear helix-loop-helix proteins, SCBP s, have been demonstrated to bind to the 5′-border of the salivary-specific cAMP-responsive elements and to activate reporter gene expression in the cotransfection assays (15). Recently, we have taken a transgenic approach to locate the regulatory regions that are essential for tissue-specific and inducible expression of rat PRP gene, R15, in vivo. The expression profiles of 18 independent transgenic lines harboring fusion constructs containing 10-, 6-, or 1.7-kb contiguous R15 5′-flanking region, respectively, and the CAT reporter gene were analyzed (16). Our data indicate that (i) the 6-kb R15 5′-flanking region is sufficient to reproduce the correct spatio-temporal transgene regulation in vivo; (ii) there is no noticeable difference among the expression profile of independently established transgenic lines that harbor 10 and 6 kb of R15 5′-flanking region; and (iii) the R15 5′-DNA sequences up to −1.7 kb relative to transcription start site, which contain the previously identified cis-elements in proximal promoter including the salivary-specific cAMP-responsive elements, fail to confer Ipr/tannin inducibility to the transgene expression in the salivary glands (16). These observations suggest that the distal regulatory elements located between the −6 and −1.7 kb of R15 5′-flanking region are essential to recapitulate the correct spatial and inducible R15 expression pattern.
in vivo. Thus, we have designated this 4.3-kb (−6 to −1.7) fragment as the R15 salivary-specific and Ipr/tannin-dependent regulatory region (SITR).

Although several trans-acting factors have been described as important for regulating the PRP proximal promoter activity (15, 17), factors mediating the response via cis-elements in the distal SITR are yet to be identified. In the present study, we have further investigated the molecular mechanisms governing the tissue-specific PRP induction by cAMP in vivo and in vitro. Analyses in the transgenic mice reveal that the PRP promoters are inducible by both Ipr and dietary tannins in the salivary glands only when the DNA fragment located between −2.4 and −1.7 kb of the SITR is part of the transgene. With heterologous promoter constructs and transient transfection in parotid cells, we further demonstrate here that the minimal control region of the R15 SITR extends from −1995 to −1812 nucleotides relative to the transcription start site.

Classically, transcriptional activation in response to increased intracellular cAMP levels has been found to be regulated by the cAMP response element (CRE), which binds trans-acting factors such as the CRE-binding protein/activating transcription factors family. The function mediated by members of this family in the cAMP/protein kinase A signal transduction pathway has been extensively characterized (reviewed in Ref. 18). However, sequencing analysis indicates that there is no consensus CRE site in this 184-bp (−1995 to −1812 nucleotides) distal regulatory element. Instead, several hexameric elements that are either identical or homologous to an estrogen or retinoic acid receptor half-site (reviewed in Refs. 19, 20) are located within this element. Members of the nuclear orphan receptor superfamily, including NGFI-B, NURR1, and SF-1, are demonstrated to bind to each response element as a monomer and activate specific gene expression (19, 20). To address the functional role(s) of these hexamer sequences in regulating salivary PRP expression, we performed the cotransfection experiment. The results from these analyses suggest that the NGFI-B or a closely related factor, NURR1, is involved as a major trans-regulator in the tissue-specific induction of PRP expression brought about by chronic Ipr injection or tannin feeding through cAMP as a second messenger.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transient Transfection**—The immortalized normal rat parotid acinar cell lines, Pa-3 and Pa-4, were obtained from Dr. D. O. Quissell (University of Colorado Health Science Center). These cells were plated on primaria culture dishes (Falcon) in Dulbecco’s modified Eagle’s medium/F12 (1:1) medium supplemented with 2.5% fetal bovine serum, insulin (5 µg/ml), transferrin (5 µg/ml), epidermal growth factor (25 ng/ml), hydrocortisone (1.1 µM), glutamate (5 mM) and were maintained in a humidified atmosphere containing 5% CO2 and 95% air at 35 °C. NIH3T3 cellsweregrown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37 °C in 5% CO2. All culture media contained 60 µg/ml kanamycin monosulfate. Transient transfection of the plasmid constructs and mutant constructs was confirmed by DNA sequencing. The CMV-NGFI-B, CMV-NURR1, and CMV-SF-1 (provided by J. Milbrandt, Washington University, St. Louis, MO) contain the orf CNS virus thymidine kinase (TK) promoter which contains a herpes simplex virus thymidine kinase (TK) promoter with a complete BglII BamHI (−2446 to −1742) fragment, in the sense orientation, from the R15 5′-flanking region into the BamHI site of the pBl2CAT, which contains a herpes simplex virus thymidine kinase promoter (−109 to +51) upstream of the CAT gene (21). The following restriction sites were used to construct the 5′-deletion mutants of (−2.4/−1.7)TK-CAT: SpI (−2331), MunI (−1994) and NheI (−1812). The 3′-deletion mutants of (−2.4/−1.7)TK-CAT were generated by using the Erase-A-Base system (Promega). The site-directed mutagenesis on each hexamer site of (−1995/−1812)TK-CAT was carried out using the TransforMax Escherichia coli-based directed mutagenesis kits from Clontech. The accuracy of all the plasmid constructs and mutant constructs was confirmed by DNA sequencing. The CMV-NGFI-B, CMV-NURR1, and CMV-SF-1 (provided by J. Milbrandt, Washington University, St. Louis, MO) contain the orphan nuclear receptor NGFI-B, NURR1, and SF-1, respectively, under the CMV promoter/enhancer control. The dominant negative mutant NGFI-B (−235/−195) was constructed by deleting by the transcriptional activation domain (amino acids 25–195) of NGFI-B (22). All plasmid DNAs were purified on QIAGEN columns according to the manufacturer’s instructions.

**Production and Characterization of Transgenic Mice**—Transgenes −3.8PRP/CAT, −2.4PRP/CAT, and −8.2Δ1.3PRP/CAT were excised from their corresponding plasmids by HindIII and KpnI digestions. The DNA fragments were purified from agarose gel by GeneClean procedure (Bio-101), and 1–2 plicoliters of DNA were injected at a concentration of 2 ng/µl into pronuclei of FVB/N or B6/CBA F2 mouse zygotes, as described previously (16). Transgenic founders were identified by dot blot hybridization or polymerase chain reaction with a primer pair specific for the CAT transgene, using DNA prepared from the tail tissues of the pups at the age of 3–4 weeks. For transgene copy number determination, DNA was prepared from livers of independent lines of transgenic mice. Southern blot analyses of the liver DNA showed that, in all cases, the transgenes in each transgenic mouse line were stably integrated at a single site. Copy numbers of PRP/CAT transgene in each line were estimated by comparing the blot intensity of the CAT hybridization band to that of known standards. The regulation of transgene expression was studied essentially under three metabolic conditions, control (fed with Purina Lab Chow only), Ipr-injected (daily injection of isoproterenol for 10 days), and tannin-fed (fed with a 6% tannin-containing diet for 6 days) as described previously (16).

**Reporter Assays**—Mouse tissues (0.1–0.2 g) were homogenized in 1 ml of buffer A (15 mM Tris-HCl (pH 8.0), 60 mM KCl, 15 mM NaCl, 2 mM EDTA, 0.15 mM spermine, 1 mM dithiothreitol, and 0.4 mM phenylmethylsulfonyl fluoride). After three cycles of freezing and thawing, homogenates were heated at 65 °C for 10 min. The tissue extracts were cleared by centrifugation (12,000 × g for 10 min at 4 °C), and protein concentrations in the supernatants were determined by a Bio-Rad Kit. CAT activity was determined, as described previously, using 0.5 mCi of [14C]chloramphenicol in a 200-µl reaction mixture. For determination of CAT activity, cleared cell extracts were diluted into the linear range of the reaction (1–40% conversion) in order to quantitate the fold of induction in different experimental conditions. The percent conversion is calculated as the total acetylated products over the input of [14C]chloramphenicol.

Cell extracts were prepared by lysing the transfected cells in reporter lysis buffer (25 mM Tris phosphate (pH 7.8), 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N,N,N′-tetraacetic acid, 1% glycerol, and 1% Triton X-100; Promega), as suggested by the manufacturer. For the luciferase assay, the supernatant of the cell lysate (10 µl) was mixed with 100 µl of assay buffer (20 mM Tricine, 1.07 mM (MgCO3)2Mg(OH)2), 5 mM NaCl, 2.67 mM MgSO4, 0.1 µM EDTA, 33.3 mM dithiothreitol, 270 µM coenzyme A, 470 µM luciferin, and 530 µM ATP), and light emission was measured in a model 20 luminometer (Turner Design). Cell extracts normalized to the same luciferase activity were used in the CAT assays. CAT activity was assayed by thin layer chromatography as described above.

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D. O. Quissell, K. A. Borzem, R. S. Redman, J. M. Camdam, and J. P. Turner, submitted for publication.
Unique DNA fragments were inserted in the sense orientation at a
and a 0.7-kb (2.2-kb) 
ated three subfragments from the 4.3-kb (the essential regulatory region(s) within the SITR, we gener-
flanking region as the putative SITR. In order to further locate
expression, we have designated the
region in modulating
Enhancer Activity in Transgenic Mice---
To investigate the putative role of the
PRP/CAT transgenic mice harboring the 2.1-kb
activity by both Ipr injection and tannin feeding was observed in
the parotid and submandibular glands of all three independently estab-
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the parotid and submandibular glands of all three independently estab-
lished

3.8- to 1.7 region upstream of the −1.7PRP/CAT (Fig. 1) were established. Among these three transgenic lines, a moderate CAT activity was detected in the parotid glands of one of the untreated mouse lines (Fig. 2) but not in the other two transgenic lines (Table I). However, a marked induction of CAT activity by both Ipr injection and tannin feeding was observed in the parotid and submandibular glands of all three −3.8PRP/CAT transgenic lines (Fig. 2). Notably, high levels of induction by either Ipr or dietary tannins were reproducibly observed in the submandibular glands of all three independently established −3.8PRP/CAT transgenic lines. This unusual submandibular induction of transgene expression by Ipr and dietary tannins is different from the parotid-specific expression profile observed in the −6PRP/CAT transgenic mice (Fig. 2). An analysis of CAT activities in nine other −3.8PRP/CAT founder mice revealed that about one-third exhibited basal (uninduced) CAT expression in the parotid glands. Taken together, these data indicate that the 2.1-kb (−3.8 to −1.7) region not only mediates a high level of transgene induction by catecholamine Ipr and dietary tannins in both parotid and submandibular glands, but also directs a detectable basal transgene expression in the parotid glands in vivo (Fig. 2 and Table I).

A further 5′-deletion beyond −3.8 kb of R15 5′-flanking region was carried out, and two −2.4PRP/CAT transgenic lines were derived from the construct that contains only 0.7-kb (−2.4

### RESULTS

A 0.7-kb Fragment of the R15 5′-Flanking SITR Confers Full Enhancer Activity in Transgenic Mice—The −6 to −1.7 kb R15 5′-flanking region appears to be crucial for directing Ipr/tannin-
dependent transgene expression exclusively in the parotid glands of 18 independent transgenic lines that have been previously described (16). Based on the profiles of transgene expression, we have designated the −6 to −1.7 kb of R15 5′-flanking region as the putative SITR. In order to further locate the essential regulatory region(s) within the SITR, we generated three subfragments from the 4.3-kb (−6 to −1.7) SITR, a 2.2-kb (−6 to −3.8) fragment, a 2.1-kb (−3.8 to −1.7) fragment, and a 0.7-kb (−2.4 to −1.7) fragment. These three individual DNA fragments were inserted in the sense orientation at a unique BamHI site 5′ to the −1.7PRP/CAT (Fig. 1), and the reporter CAT activity from each construct was explored.

The expression profiles from each transgene in tissues under different experimental conditions were analyzed by the CAT assays, as shown in Fig. 2 and summarized in Table I. All except three of the transgenic lines have more than one copy of transgenes integrated at a single integration site in a head to tail arrangement. Four independent −6Δ2.1PRP/CAT transgenic lines harboring transgenes with an internal deletion of a 2.1-kb fragment (−3.8 to −1.7) from the 6-kb R15 5′-flanking region (Fig. 1) were established. CAT activity was not detected in any of the 11 tissues tested including brain, parotid, submandibular glands, and liver from both control and treated mice, and all four independent lines displayed a similar expression profile. This observation indicates that the 2.1-kb internal deletion can completely abolish the observed inducible salivary transgene expression from the −6PRP/CAT construct in vivo.

To investigate the putative role of the −3.8- to −1.7-kb region in modulating R15 promoter activity, three independent lines of −3.8PRP/CAT transgenic mice harboring the 2.1-kb

![Schematic diagram of PRP/CAT transgene construct.](image)

**Fig. 1.** Schematic diagram of PRP/CAT transgene construct. Top line is a schematic diagram of rat proline-rich gene, R15, and the restriction enzyme sites are indicated above. Three exons are shown as black boxes. The numbers indicating the distance in kb from the transcription initiation site (→) are shown underneath the second horizontal line, except that 2* represents nucleotide +2. Varying lengths of genomic fragments derived from R15 cosmid were inserted upstream of the promotorless chloramphenical acetyltransferase (CAT) structural gene to create constructs used in the transgenic animal studies. B, BamHI; Bg, BglII; E, EcoRI; N, NcoI; P, PstI.

**Fig. 2.** Representative expression profile of PRP/CAT transgene in transgenic mice. Protein extracts were prepared from indicated tissues of −6PRP/CAT, −6Δ2.1PRP/CAT, −3.8PRP/CAT, −2.4PRP/CAT, and −1.7PRP/CAT transgenic mice fed with Purina Lab Chow (Control), injected daily with 1 mg of dl-isoproterenol for 10 days (J) or fed with a diet containing IS-8260 high tannin content sorghum for 6 days (T). The CAT assays were performed with 100 µg of soluble protein from each sample, and the reaction time is for 17 h in order to demonstrate low level or no transgene expression in nonsalivary tissues of transgenic mice. Eleven tissues (brain, heart, kidney, liver, lung, muscle, parotid gland, submandibular gland, spleen, thymus, and trachea) were evaluated. Part of the tissue-survey results from −6PRP/
CAT and −1.7PRP/CAT transgenic mice have been published previ-
ously (16), and only results from selected CAT assays were shown here. Br, brain; Li, liver; Pa, parotid gland; Su, submandibular gland; Th, thymus; and Tr, trachea.

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Tissue-specific and Inducible Gene Expression-mediated by NGFI-B

**Summary of PRP/CAT transgenic studies**

| Transgene | Foundera | Transgenic line | Transgene copy | Parotid | Submandibular | Ectopic exp. |
|-----------|----------|----------------|---------------|---------|---------------|-------------|
| -6PRP/CAT | 5F       | -6PRP/CAT-01   | 26            | -       | +             | -           | -           |
|           | 2M       | -6PRP/CAT-02   | 1             | -       | +             | -           | -           |
|           |          | -6PRP/CAT-03   | 2             | -       | +             | -           | -           |
|           |          | -6PRP/CAT-04   | 2             | -       | +             | -           | -           |
| -6(Δ2.1)PRP/CAT | 4M | -6(Δ2.1)PRP/CAT-01 | 2 | - | + | - | - |
|           |          | -6(Δ2.1)PRP/CAT-02 | 3 | - | - | - | - |
|           |          | -6(Δ2.1)PRP/CAT-03 | 1 | - | - | - | - |
| -3.8PRP/CAT | 7F | -3.8PRP/CAT-01   | 4 | - | + | + | + |
|           | 5M       | -3.8PRP/CAT-02   | 1 | - | + | + | + |
|           |          | -3.8PRP/CAT-03   | 4 | - | + | + | + |
| -2.4PRP/CAT | IF  | -2.4PRP/CAT-01   | 6 | - | + | - | - |
|           | IF       | -2.4PRP/CAT-02   | - | + | - | - | - |
| -1.7PRP/CAT | IF  | -1.7PRP/CAT-01   | 45 | - | + | - | - |
|           | 1M       | -1.7PRP/CAT-02   | 21 | - | + | - | - |

a F, female; M, male.

The inducible expression profiles of CAT transgene in the parotid glands of these mice were qualitatively and quantitatively similar to that observed in the -3.8PRP/CAT transgenic mice. However, the levels of induced transgene expression in the submandibular glands were dramatically reduced to barely detectable (Fig. 2). This observation suggests that the DNA sequences from -3.8 to -2.4 kb of R15 are involved in conferring the Ipr/tannin inducibility to the transgene expression in the submandibular glands. It is also possible that the DNA sequences in the -6 to -3.8-kb region may have the ability to negatively modulate the inducible CAT expression in the submandibular glands of -6PRP/CAT mice (see “Discussion” below). However, the nature of these activities is still unclear.

**Comparison of Relative CAT Activities and Inducibilities in the Salivary Glands of Series of PRP/CAT Transgenic Mice—** Our analyses of CAT transgene expression in the salivary glands indicate that the observed tissue-specific and inducible transgene expression can be achieved with constructs containing 10-, 6-, 3.8-, and 2.4- but not 6 (Δ2.1)- and 1.7-kb of R15 5’-flanking region (Fig. 2). In some transgenic lines, ectopic transgene expression was observed in the brain, thymus, and trachea, as shown in Fig. 2 and summarized in Table I; however, the patterns of ectopic expression are not always consistent. There was also noticeable variation in the levels of transgene expression among different transgenic lines derived from the same construct, and these differences cannot be explained entirely by the different copy numbers of the integrated transgenes. It has been suggested that the copy number-independent transgene expression may result from the mosaicism of transgenic mice when founder transgenic mice were used in the study. However, the mice analyzed here were either N1 or N2 offspring, and there was only one integration site in each established transgenic line (Table I). It is tempting to speculate that not all the mice responded to the Ipr/tannin stimulation and expressed the transgenes equally. A similar observation has also been reported in other transgenic system.

In order to quantitatively analyze the expression level from each transgene, the average CAT activity per copy of transgene from all six different PRP/CAT constructs was analyzed and compared (Fig. 3). The average CAT activities in both parotid and submandibular glands from pooled (at least three mice) control, Ipr-injected, or tannin-fed mice of each independent line were compared after being normalized with the transgene copy numbers. One relative unit of CAT activity is defined as 1% chloramphenicol conversion per copy of transgene when 50 μg of soluble parotid protein extract from Ipr-injected -6PRP/CAT-01 mice was incubated at 37°C for 1 h. Serial dilutions were made in the cases of protein extracts from treated -3.8PRP/CAT and -2.4PRP/CAT transgenic mice to obtain a linear range of CAT activities for the purpose of comparison. When 6 kb of the R15 5′-flanking region was included in the transgene construct, the basal CAT activities in both parotid and submandibular glands are negligible (<1 unit, Fig. 3), which reflects the endogenous R15 expression in the salivary glands of PRP/CAT transgenic mice.
glands of control mice (16). Induced relative CAT activities were lower in the submandibular glands (Ipr, 30 ± 11 units; tannin, 29 ± 11 units; Fig. 3). An internal deletion of 2.1-kb (−3.8 to −1.7) DNA fragment from the −6PRP/CAT construct caused a loss of the Ipr/tannin-inducible CAT transgene expression in the salivary glands (<1 unit) of −6(D24.1)PRP/CAT transgenic mice (Figs. 2 and 3). A deletion of 5′-flanking region from −6 to −3.8 kb resulted in a moderate increase in the average basal CAT activity detected in the parotid glands (control, 3 ± 1 units, Fig. 3) of −3.8PRP/CAT transgenic mice. Also, the induced relative CAT activities in the parotid glands of −3.8PRP/ CAT mice (Ipr, 3863 ± 565 units; tannin, 2800 ± 529 units; Fig. 3) were at least 70-fold higher than that in the −6PRP/CAT mice.

This comparison substantiates the assumption that the DNA sequences located between −6 and −3.8-kb region are able to negatively modulate the inducible expression mediated via SITR. On the other hand, a 1.4-kb deletion, extended from −3.8 to −2.4 kb of the R15 5′-flanking region, caused the salivary basal activity to be undetectable (<1 unit; Fig. 3), but the remaining 2.4-kb 5′-flanking region was able to elicit an induction of transgene CAT activity by Ipr and tannin in the parotid glands to a level comparable with that observed in the −3.8PRP/CAT transgenic mice (Figs. 2 and 3). In contrast, further deletion to −1.7 kb abolished the induction in the salivary glands (<1 unit) by both Ipr and dietary tannins, despite that a moderate basal CAT activity was detected in the parotid and submandibular glands of the control −1.7PRP/CAT transgenic mice (Figs. 2 and 3). Taking together the results from PRP/CAT series of transgenic mice that we reported here and previously (16), we conclude that the DNA sequences extended from −2.4 to −1.7 kb of R15 5′-flanking region is indispensable for the parotid-specific and Ipr/tannin-inducible PRP expression in vivo.

**Characteristics of the R15 Distal Enhancer**—One of the two previously identified parotid-specific DNase I-hypersensitive sites in the R15 5′-flanking region is also mapped to this −2.4 to −1.7-kb DNA region (16). Hence, this 0.7-kb genomic fragment and its 5′- and 3′-deletion mutants were further analyzed by generating heterologous TK promoter/reporter constructs and subsequent transient transfection assays (Fig. 4). The well-differentiated parotid acinar Pa-4 cells, less-differentiated parotid Pa-3 cells, and NIH 3T3 fibroblast cells were used as host cells for transfection. Transfection efficiencies among experiments were normalized by cotransfection of pSV2-luciferase reporter gene. A direct comparison of experimental data from Pa-4, Pa-3, and NIH 3T3 cells was difficult because transfection efficiencies differed by a factor greater than 10, as judged from the luciferase activity. Since it has been demonstrated that the Ipr-mediated PRP induction is via cAMP as a second messenger (14), the cAMP inducibilities of various constructs after transient transfection into different host cells were monitored in order to delineate the putative cAMP response element(s) within the 0.7-kb (−2.4 to −1.7) fragment.

As shown in Fig. 4, expression from (−2.4/-1.7)TK-CAT was greatly stimulated by the cAMP in Pa-4 cells but not in Pa-3 and NIH 3T3 cells. This observation is consistent with the notion that a high level of inducible PRP expression is exclusively associated with the fully differentiated salivary acinar cells (6, 16). Furthermore, it also indicates that one of the DNA motifs involved in dictating the salivary acinar cell specificity is located in the distal 0.7-kb region. 5′-Deletions of this 0.7-kb fragment from −2446 down to −1995 nucleotides and the 3′- to 5′-deletions from −1742 to −1928 nucleotides did not alter the cAMP inducibility on heterologous TK promoter in Pa-4 cells drastically; however, further deletions did (Fig. 4). When reporter construct G (Fig. 4) containing a limited fragment extended from −1995 to −1812 nucleotides was tested, a full cAMP inducibility comparable with that of (−2.4/-1.7)TK-CAT

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**Fig. 4. Deletion analysis of R15 upstream enhancer region.** The parotid Pa-3, Pa-4, and NIH 3T3 cells were transfected with the indicated reporter constructs, respectively, and the control plasmid pSV2-luciferase to normalize the transfection efficiency in each cell line. The transfected cells were treated with 10 μM forskolin or 1 mM CAMP for 4 h. The CAT activity in each transfected cells was determined and normalized with the luciferase activity, as described under “Experimental Procedures.” The fold induction expressed in Pa-4 (●), NIH 3T3 (●●), and Pa-3 (□) cells is calculated by dividing the CAT activity in extracts from the treated cells over that of the control cells. Error bars show the standard error for each construct in different cell lines, respectively, which are based on at least three independent assays.

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| Deletion | Fold Induction by cAMP |
|----------|------------------------|
| A        | TK CAT                 |
| B        | TK CAT                 |
| C        | TK CAT                 |
| D        | TK CAT                 |
| E        | TK CAT                 |
| F        | TK CAT                 |
| G        | TK CAT                 |
| H        | TK CAT                 |

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**Table:** Deletion analysis of R15 upstream enhancer region. The parotid Pa-3, Pa-4, and NIH 3T3 cells were transfected with the indicated reporter constructs, respectively, and the control plasmid pSV2-luciferase to normalize the transfection efficiency in each cell line. The transfected cells were treated with 10 μM forskolin or 1 mM CAMP for 4 h. The CAT activity in each transfected cells was determined and normalized with the luciferase activity, as described under “Experimental Procedures.” The fold induction expressed in Pa-4 (●), NIH 3T3 (●●), and Pa-3 (□) cells is calculated by dividing the CAT activity in extracts from the treated cells over that of the control cells. Error bars show the standard error for each construct in different cell lines, respectively, which are based on at least three independent assays.

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**Diagram:** Deletion analysis of R15 upstream enhancer region. The parotid Pa-3, Pa-4, and NIH 3T3 cells were transfected with the indicated reporter constructs, respectively, and the control plasmid pSV2-luciferase to normalize the transfection efficiency in each cell line. The transfected cells were treated with 10 μM forskolin or 1 mM CAMP for 4 h. The CAT activity in each transfected cells was determined and normalized with the luciferase activity, as described under “Experimental Procedures.” The fold induction expressed in Pa-4 (●), NIH 3T3 (●●), and Pa-3 (□) cells is calculated by dividing the CAT activity in extracts from the treated cells over that of the control cells. Error bars show the standard error for each construct in different cell lines, respectively, which are based on at least three independent assays.
shown in Fig. 4 and resulted in a marked increase of the constructs mimicked the induction mediated by cAMP as showed that cotransfection of NGFI-B and NURR1 expression vectors in well-differentiated parotid Pa-4 cells. Our data indicated that cotransfection of NGFI-B and NURR1 expression vectors into NIH 3T3 cells transfected with all these constructs. As expected, the expression of TK-CAT construct was not affected by cAMP treatment in the Pa-4 cells, which serves as a negative control for the analysis. Therefore, this 184-bp fragment, extended from −1995 to −1812 nucleotides (Fig. 4). There was no cAMP inducibility detected from the Pa-3 and NIH 3T3 cells transfected with all these constructs. As expected, the expression of TK-CAT construct was not affected by cAMP treatment in the Pa-4 cells, which serves as a negative control for the analysis. Therefore, this 184-bp fragment, extended from −1995 to −1812 nucleotides, in the distal R15 5′-flanking region might function as an inducible enhancer, based on its ability to stimulate the heterologous TK promoter activities in transfected parotid acinar cells and the results above from transgenic studies.

**NGFI-B Transactivates the Expression of (−1995/−1812)-TK-CAT**—A homology search for putative transcription factor-binding sites in the 184-bp fragment was performed using the Find Patterns Algorithm of the CGG software package Release 8. The transcription factor sites data base (25). A CRE-like site at −1862 nucleotides (TGAGGTCA) matching 7/8 of the consensus CRE site (TGAAGCTA) was revealed, as shown in Fig. 5. However, cotransfection experiment with pSG-CRE-binding protein expression plasmids suggested that this CRE-like site may not be functional. A 12-O-tetradecanoylphorbol-13-acetate response element-like site (TGAGGTCA) was identified at position −1892 nucleotides to match 6/7 of the consensus sequence (TGAAGCTA), which might be involved in modulating the basal activity from this 184-bp fragment. The data base analysis also identified two perfect hexameric AGGTCA sequences at positions −1891 nucleotides and −1914 nucleotides, and two less-perfect hexameric AAGTCA sequences at positions −1847 nucleotides and −1836 nucleotides (Fig. 5). The two AAGTCA sequences are arranged as direct repeats spaced by five nucleotides (DR + 5). Various configurations of the hexameric AGGTCA sequences, especially the direct repeats, have been shown to be potent response elements for homodimeric and heterodimeric steroid/thyroid superfamily nuclear receptors (reviewed in Ref. 26). In contrast, nuclear orphan receptors NGFI-B, NURR1, and SF-1 can bind to a similar element but more promiscuously, failed to stimulate the (−1995/−1812)-TK-CAT reporter activity. As expected, transfection with vector alone (pCMV) did not stimulate the expression of the reporter construct (not shown), and cotransfected NGFI-B had no effect on the expression from the parental TK-CAT (Fig. 6). To further verify the role of NGFI-B in modulating salivary PRP expression, an expression construct harboring the dominant negative mutant NGFI-B (Δ25–195), which lacks the activation domain, was cotransfected with (−1995/−1812)TK-CAT reporter construct in Pa-4 cells. As shown in Fig. 6, the expression of NGFI-B (Δ25–195) mutant did not transactivate the (−1995/−1812)-TK-CAT expression. To test the dominant negative activity, triple transfection experiments with wild-type and mutant NGFI-B expression constructs and the (−1995/−1812)-TK-CAT reporter construct were performed. Cotransfection of the mutant NGFI-B (Δ25–195) expression construct decreased NGFI-B transactivation to near background levels even at one-eighth the input level of pCMV-NGFI-B (Fig. 6) and also abolished the cAMP-induced (−1995/−1812)-TK-CAT reporter activity in the Pa-4 cells (not shown). Hence, the orphan nuclear receptor NGFI-B or a closely related factor such as NURR1 is involved in modulating the activity from the distal 184-bp (−1995 to −1812 nucleotides) fragment identified in the R15 5′-flanking region.

**Mutations in AGGTCA and AAGTCA-like Sites Abolish the Stimulating Activity Mediated by NGFI-B**—To test the significance of the two identified AGGTCA sites and the DR + 5 AGGTCA-like site (Fig. 5) on the observed NGFI-B transactivation activity, additional mutation constructs (M1 to M7) were made. These constructs carrying site-directed mutations were prepared and evaluated in combination with NGFI-B expression construct in the Pa-4 cells. As illustrated in Fig. 7, while a single mutation on an individual site had almost no effect on the observed transactivation by NGFI-B, triple mutations on all three sites resulted in a loss of more than 85% of the wild-type activity. Interestingly, double mutations on any two of the three sites impaired the NGFI-B transactivation activity.

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3 H. H. Lin and D. K. Ann, unpublished observations.
to different degrees. Hence, it is conceivable to speculate that the maximal stimulating activity mediated by NGFI-B in the parotid cells requires all three putative NGFI-B sites. Taking together, we conclude that the transcriptional stimulation from the 184-bp (1995 to −1812 nucleotides) distal fragment is highly dependent on the nuclear orphan receptor, NGFI-B.

### DISCUSSION

Extremely high inducibility of the tissue-specific PRP R15 expression, under experimental conditions that raise the intracellular cAMP levels, has prompted a detailed analysis of the regulatory elements of this gene. The tissue-specific gene expression is conferred in part by binding of specific transcriptional regulatory proteins to the proximal and distal regulatory elements (reviewed in Refs. 27, 28). In an effort to define the regulatory network that coordinately controls the tissue-specific and inducible gene expression, we undertook the characterization of the regulatory elements and their cognate binding factors. Previously, we reported that, in contrast to findings obtained from the transient transfection study, the expression patterns of the reporter transgene in vivo when driven by the 1.7-kb R15 5′-flanking region hardly reflect the endogenous R15 gene regulation (16). The aim of this study is to investigate the role(s) of regulatory regions that are located upstream of −1.7 kb in the spatio-temporal regulation of the highly characteristic expression pattern of R15. Three separate regions derived from previously designated R15 SITR extended from −6 to −1.7 kb were functionally analyzed by the transgenic approach. The results indicate the presence of a 0.7-kb region (−2.4 to −1.7) immediately upstream of −1.7 kb that enhances the induced CAT activity in the parotid glands by more than 30-fold when compared with that from the control.

Furthermore, results from the deletion analyses suggest that important regulatory sequences are contained within a 184-bp (1995 to −1812 nucleotides) fragment, which cis-activates the heterologous TK promoter in a cAMP-dependent manner after transiently transfected into Pa-4 cells. We have identified three NGFI-B sites within the distal 184-bp enhancer fragment as essential motifs mediating the stimulatory effect. This conclusion is based on several observations: (i) pCMV-NGFI-B expression construct stimulates the activity of TK promoter that linked to the 184-bp enhancer fragment; (ii) expression of a dominant negative mutant pCMV-NGFI-B(Δ25−195) construct decreases NGFI-B transactivation to almost background levels even transfected at one-eighth the amount of the cotransfected wild-type pCMV-NGFI-B construct; and (iii) mutations on the three NGFI-B sites completely abolish the NGFI-B-mediated transcriptional activation.

Our findings from site-specific mutation analyses implicate that several NGFI-B proteins bind to closely clustered NGFI-B binding sites in the R15 distal regulatory region. Many naturally occurring transcription factor binding sites are found in clusters (29, 30). For example, the TTF-1 binding sites in the human lung surfactant protein B gene are arranged in a cluster configuration (29). While the exact biological significance of these TTF-1 binding site clusters in the human surfactant B gene is still elusive, it has been suggested that the clusters of binding sites may serve to increase the occupancy of the cis-elements by the mechanism of cooperative binding or to provide potential interactive surfaces for the activator/coactivator. Since NGFI-B protein is transiently induced with a short half-life (31, 32, 34, 35), it is tempting to speculate that the clustering of NGFI-B binding sites in the distal regulatory region may facilitate the inducible R15 expression through similar mechanisms.

NGFI-B was originally identified as an immediate-early gene product rapidly activated by a variety of stimuli and belongs to the steroid/thyroid hormone receptor superfamily (31, 32). Because no ligand has been identified for NGFI-B, it is also categorized as a member of the expanding groups of orphan nuclear receptor (33). NGFI-B is transcriptionally active in many cell types as a monomer (34, 35). More recent publications show that NGFI-B and the related orphan nuclear receptor NURR1 can heterodimerize with 9-cis-retinoic acid receptor RXR (36, 37). This heterodimerization allows the NGFI-B to activate gene transcription in a ligand-dependent manner via retinoic acid response elements, which are composed of direct repeats separated by five nucleotides (DR + 5). The heterodimerization between NGFI-B and RXR is interesting because site C in R15 distal enhancer (Fig. 5) shares similarity to the DR + 5 retinoic acid response elements. However, our preliminary data suggest that the 9-cis-retinoic acid has no effect on the regulation of endogenous PRP expression and on the transfected reporter construct activity in the salivary cells (not shown). Additionally, NGFI-B is suggested to be the mediator of hormonal and neurological responses in the hypothalamic-pituitary-adrenocortical axis (38), as well as implicated in the control of steroidogenic enzyme expression in the adrenal glands (39).

It has been demonstrated that NGFI-B binds with high affinity to an AAAGTCA core site (40). This NGFI-B response element contains a classical AGGTCA hexamer site, which is identified in the 184-bp fragment of R15, preceded by two adenines (40). A region immediately C-terminal to the DNA-binding domain of NGFI-B, the A box, is required for the selective recognition of the two A/T base pairs at the 5′-end of NGFI-B response element (22). However, all three NGFI-B transactivation sites identified in R15 differ from the consensus NGFI-B response element either by the first two nucleotides preceding the hexameric sequence or by the second nucleotide of the hexamer (Fig. 5). Thus, it is conceivable that the cis-mediation by the R15 NGFI-B sites may not be so straightforward. We observed that the oligonucleotides corresponding to the sequence of any single identified R15 distal NGFI-B site by itself in a one-copy or two-copy configuration failed to cis-activate the heterologous TK promoter by more than 4-fold when cotransfected with NGFI-B. The lack of stimulation by a single NGFI-B site implies that the clustering configuration of these R15 NGFI-B sites is essential for the presumably low
affinity interaction with NGFI-B. Also, it is likely that the presence of the flanking sequences between each site is critical for the transactivation mediated by NGFI-B via the 184-bp regulatory region. Further experiments are needed to elucidate the role of the rest of the context of this fragment in mediating the inducible and tissue-specific R15 gene expression.

Modification, in particular phosphorylation/dephosphorylation, of transcription factors is a frequent event of transcriptional activation (reviewed in Ref. 41). Davis and Lau (38) have shown that cAMP regulates the NGFI-B activity in two ways. First, cAMP can increase the NGFI-B level by transcription activation, and second, cAMP alters the phosphorylation state of NGFI-B to enhance its activity. In particular, cAMP increases the phosphorylation at the N terminus and causes the hypophosphorylation of serine 350 of NGFI-B. Nevertheless, it is unclear how these modifications contribute to the salivary gland transcription factor activity.

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