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The Golden Hamster Aphrodisin Gene

STRUCTURE, EXPRESSION IN PAROTID GLANDS OF FEMALE ANIMALS, AND COMPARISON WITH A SIMILAR MURINE GENE*

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Hans-Jürgen Mägert‡, Alexandra Cieslak, Ozan Alkan, Bernhard Lüscher§, Wolfgang Kauffels¶, and Wolf-Georg Forssmann

From the Lower Saxony Institute for Peptide Research, D-30 625 Hannover, Germany and Departments of §Molecular Biology and ¶Gynecology and Obstetrics, Hannover Medical School, D-30625 Hannover, Germany

The so-called lipocalins are a family of extracellular proteins that are known to typically fulfill tasks as transport proteins for small hydrophobic molecules. However, in the last decade, a large diversity has been described concerning their functions, for example as enzymes, immunomodulators, or proteins involved in coloration and pheromone action. Aphrodisin belongs to those lipocalins, which are of significant importance for the pheromonal stimulation of copulatory behavior in male hamsters. We recently succeeded in characterizing the corresponding cDNA and demonstrated the expression of the aphrodisin gene in the vagina, uterus, and Bartholin's glands of female hamsters. Here we report the structure of the aphrodisin gene and the functionality of its promoter region. We further compare the aphrodisin gene to the related gene for mouse odorant-binding protein 1a, indicating similar functions of their products. As a novelty, we show that the aphrodisin gene, in addition to the above-mentioned tissues, is also expressed in female hamster parotid glands. In contradiction to the results expected, we finally demonstrate that aphrodisin already occurs in vaginal discharge before the female animals reach fertility. These findings may lead to the identification of as yet unknown aphrodisin functions.

Lipocalins are a family of proteins that typically exhibit a molecular mass in the range of ~17–30 kDa. Their main function seems to be the transport of low molecular mass hydrophobic substances such as retinol, progesterone, odorants, and even pheromones within hydrophilic environments. For this purpose, lipocalins exhibit a certain tertiary structure, which is comparable to a coffee filter, containing an apolar binding pocket for specific ligands. This structure is formed by two sets of four antiparallel β-strands arranged in two orthogonally stranded β-sheets (for review, see Ref. 1). Besides their functions as transporter molecules, in the last decade manifold additional tasks have been described, including functions as enzymes (2, 3), gustatory proteins (4, 5), immunomodulators (6), cell regulators (7–9), and proteins involved in the coloration of animals (10). Depending on the existence of all or just a subset of three conserved amino acid stretches, lipocalins are meanwhile divided into subgroups of the so-called kernel and outlier lipocalins (1). Surprisingly, even bacterial lipocalins have recently been discovered as members of the latter subgroup (11, 12).

Another prominent member of the outlier lipocalins is aphrodisin, which was originally isolated from hamster vaginal discharge, where it occurs in relatively high concentrations, such as 100 μg/20 mg of discharge (13, 14). Aphrodisin was demonstrated to be essential for the pheromonal stimulation of copulatory behavior in male hamsters acting via the vomeronasal organ (15), which is located within the nasal septum of a large number of different vertebrates. Although it may be degenerated, humans also seem to possess a functionally active vomeronasal organ (16–18). At the present time it is still not known whether aphrodisin itself or the combination with a low molecular mass ligand is necessary for pheromonal activity (19). However, the ability of purified aphrodisin to modulate the production of the second messenger inositol-1,4,5-triphosphate in membranes of the male hamster's vomeronasal organ could already be demonstrated (20).

To obtain nucleotide sequence information for the construction of hybridization probes and PCR[1] primers to facilitate the identification of related systems in species other than the hamster, we have cloned and characterized the aphrodisin cDNA. We further demonstrated the high level expression of the corresponding gene in vaginal tissue, several segments of the uterus, and the Bartholin's glands and characterized part of its promoter region (21, 22). In this paper, we report the structure of the entire aphrodisin gene and show the functionality of its promoter. As a novelty, we describe its expression in the parotid glands of female but not of male golden hamsters. In contradiction to the results expected for a copulation-stimulating pheromone, we demonstrate the significant occurrence of aphrodisin in golden hamster vaginal discharge before the female animals reach fertility. In addition, we describe the gene for mouse odorant-binding protein 1a (MMOBP1A), which possibly exhibits a function comparable with that of aphrodisin. These results may in the future allow the discovery of so far unknown functions of aphrodisin, the characterization of related proteins and their genes in mammalian species other than hamster, and the investigation of regulatory mechanisms of the gene.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ225170.

‡ To whom correspondence should be addressed: Lower Saxony Institute for Peptide Research, Feodor-Lynen-Strasse 31, D-30 625 Hannover, Germany. Tel.: 49-511-546-6310; Fax: 49-511-546-6102; E-mail: HJ-Maegert@gmx.de.

[1] The abbreviations used are: PCR, polymerase chain reaction; MMOBP1A, mouse odorant-binding protein 1a; bp, base pair; kbp, kilobase pair; RT, reverse transcription.

EXPERIMENTAL PROCEDURES

Oligodeoxyribonucleotides—The oligonucleotides listed below were purchased from Perkin-Elmer and used as PCR primers, sequencing primers, and hybridization probes (listed in 5′→3′ direction) (primers used for primer walking sequencing are not listed): AP-24, GCTTCTTGAAACAATTTATTTAATCAGT; AP-42, CTAAACACTGAAGTCAACTCCTGGAACCCAG; AP-44, CTTAGTGACTATTTATGGGCTTTTTGAATG; OZ-1, TTAAAGAGCTCACGCGTGCGGCCGCTTGGGACAGGGTGGAAGG; OZ-2, CCAAACTCGAGATCTGGTGCCTGACTTTGCTTTTCC; MAP-66, TGCTGCTTGCTTTGACTTTGGACTGGCAC; MAP-67, ATCAGCAGCAATAGCAACAGTTTTCCATGG; MAP-68, CTTACCTGTGAAAAGGAATGCAAGGAAATG; MAP-69, CCCAGTGATTGTGGTCAATGAGCACTGTCC; MAP-70, TGCAAGAAGATGGCAAGACCTACAAAACTC; MAP-71, CTCATCCACAAGTTTATAACGATTATTCCC; MAP-81, GGGAATAATCGTTATAAACTTGTGGATGAG; MAP-82, GAGAACTTAACATTTTATAGTGAGAACGTGG; MAP-83, TGTGCCAGTCCAAATGTCAAAGCAAGCAGC; MAP-84, GCCATGATGGTACAAGACTTTCTTTTCTAC; MAP-105, GATAAAATATGTCATTCCTTACTCTACTCAC; MAP-106, ACTCACTTTCTTTTACAGATTACTGTCCTG; MAPEX-1, CCTTCACAACTACTTATCTGCTTTGAGC; MAPEX-2, GAGGGAAGCTCACACTTTATAAACAC; MAPEX-3, CAACTCATATTCTAATGGTTTGTATGG; MAPEX-4, TCAGTTTAYATTYTTGATAATTTGC; MAPEX-5, GATGTAATGGTAGCAAAGAACAAACC; MAPEX-6, CAACTACAATGCTTTGGTCATAGAGGG; MAPEX-7, GTAGAACATTGAGAGTTACTGACTTTTG; and MAPEX-8, GCAGTAATTTTTTTACTATTTCAGAGATCC.

Cloning and Characterization of the Golden Hamster Aphrodisin Gene—Cloning, Southern blotting, subcloning of positive restriction fragments, and sequencing of the golden hamster aphrodisin gene were accomplished as already described (21). PCR fragments were cloned in pGEM-T vector (Promega). Nucleotide sequences were determined on both strands. In the case of PCR-generated fragments, at least three independent clones were sequenced. Comparison of obtained partial sequences with each other was performed by means of the MacMolly software package (Softgene, Berlin, Germany). Potential regulatory elements within the 5′-flanking region of the golden hamster aphrodisin gene and the related MMOBP1A gene of the mouse were detected using the MatInspector program (23) on an Apple Power Macintosh 8200/120 computer. For analysis of the functionality of different promoter fragments, luciferase reporter gene assays with human T 84 cells and different derivatives of the vector pGL2 were performed exactly as described (24). The largest of the promoter fragments used was generated by PCR (25) with the primer pair OZ-1/OZ-2 and a pUC18 derivate containing the 5′-terminal 5.7-kbp *Sst*I fragment of the gene as a template (21). Reaction conditions were as follows: 99 °C, 2 min; addition of 2.5 units of *Taq* DNA-polymerase (Boehringer Mannheim)/50-μl reaction volume at 72 °C; 95 °C, 30 s, 48 °C, 30 s, 65 °C, 5 min, 19 cycles; 95 °C, 30 s, 48 °C, 30 s, 65 °C, 10 min, one cycle. 1 μg of the abovementioned plasmid template was used/50-μl reaction mixture. The 2144-bp fragment obtained was cloned site-directed into the *Sst*I-*Xho*I sites of the vector pGL2 basic (Promega). Two additional subclones containing smaller 5′-terminally truncated insertions were generated by linearization of the clone with *Sst*I, subsequent hydrolysis with either *Pst*I or *Bpu 1102I*, generation of blunt ends using Klenow enzyme (Boehringer Mannheim), and religation.

Gene Expression Analysis—Golden hamster vaginal discharge and saliva samples were taken by rinsing the vagina or throat with physiological sodium chloride solution using a catheter. The liquid was collected in small reaction tubes by means of a funnel and stored at −20 °C. Concentration of the proteins was achieved by precipitation with a 10-fold volume of ethanol and subsequent sedimentation at 4 °C. Western blot analysis, RNA extraction, cDNA first strand synthesis, and analytical RT-PCR were performed as described recently (22).

Cloning of Fragments of the MMOBP1A Gene—High molecular mass mouse genomic DNA was a kind gift from Sigrid Wattler. Three fragments of the mouse MMOBP1A gene spanning the end of exon 1 to the beginning of exon 2, the end of exon 2 to the beginning of exon 3, and the end of exon 3 to the beginning of exon 4, were amplified using the primer pairs MAP-66/67, MAP-68/69, and MAP-70/71, respectively, 100 ng of the mouse genomic DNA in each reaction, GeneAmp *Tth* DNA-polymerase, and a model 9600 thermal cycler (both from Perkin-Elmer) under the following conditions: 94 °C, 3 min; 98 °C, 0 s, 50 °C, 30 s, 72 °C, 2 min, 39 cycles; 98 °C, 0 s, 50 °C, 30 s, 72 °C, 5 min, one cycle. Exons 1–4 of the MMOBP1A gene were amplified in the same way but using the primer pairs MAPEX-1/2, MAPEX-3/4, MAPEX-5/6, and MAPEX-7/8, respectively. 5′- and 3′-terminal fragments of the gene were amplified by means of the GenomeWalker mouse kit (CLONTECH, Palo Alto, CA) (26) according to the manufacturer's instructions with modifications. The GeneAmp XL PCR kit (Perkin-Elmer) was used without *Tth*Start antibody under the following conditions: first amplification, 94 °C, 25 s, 72 °C, 4 min, seven cycles; 94 °C, 25 s, 55 °C, 10 s, 67 °C, 4 min, 32 cycles; 67 °C for an additional 4 min; cold start; PCR primers GSP-1 and AP-1; second amplification, 94 °C, 25 s, 72 °C, 4 min, five cycles; 94 °C, 25 s, 55 °C, 10 s, 67 °C, 4 min, 24 cycles; 67 °C for an additional 4 min; cold start; PCR primers GSP-2 and AP-2. GSP-1 and GSP-2 were MAP-83/MAP-84 for the amplification of the 5′-terminal fragment and MAP-81/MAP-82 for the 3′-terminal fragment. However, because the obtained fragment did not represent the entire 3′ terminus of the gene, a second amplification step was performed with the primers MAP-105 and MAP-106, which were derived from the novel sequence information. Fragments obtained were cloned and sequenced as described above.

RESULTS

Gene Analysis—As already described, a 5.7-kbp *Sst*I fragment of the aphrodisin gene, spanning the region from the 5′ terminus down to position 5657 of the data base entry, was cloned in pBSK. To obtain an additional subclone containing the 3′-terminal remainder of the gene, Southern blotting was performed with different restriction fragments of the gene and the oligonucleotide AP-24, which was derived from the 3′ terminus of the cDNA, as a hybridization probe. A positive 1.5-kbp *Eco*RI fragment, which seemed to be large enough to overlap with the 5.7-kbp *Sst*I fragment, was subcloned in pBSK. However, the partial sequencing results obtained indicated that a further gene fragment for the connection of both subfragments mentioned above was still needed. Finally, this fragment was generated by PCR with primers derived from the 3′ terminus of the 5.7-kbp *Sst*I fragment (AP-42) and the 3′-terminal region of the 1.5-kbp *Eco*RI fragment (AP-44) and DNA of the phage clone as a template. The 2124-bp PCR fragment obtained was subcloned in pGEM-T vector, and the nucleotide sequence of the entire golden hamster aphrodisin gene was assembled by means of the primer walking sequencing strategy with subsequent sequence comparisons.

By comparison of the nucleotide sequence of the gene with the already known cDNA sequence, the following gene structure was determined: the entire aphrodisin gene spans a length of >6 kbp excluding the 5′-flanking region. It consists of seven exons, which are split by six introns. With the exception of intron 5, all exon-intron splice junctions agree well with the exon | GTRAG-intron-AG | exon rule (27). Exon 7 does not contain any coding region but just represents the main part of the 3′ untranslated region (Fig. 1).

To investigate the general importance of an aphrodisin-related pheromone system in mammals, we have tried in vain for a long time to clone the corresponding cDNAs and genes from different species using PCR primers and hybridization probes derived from the nucleotide sequence of the hamster. This intention harbors extraordinary difficulties, because proteins of the lipocalin family normally exhibit a high divergence of their amino acid sequences. As a rule, different lipocalins share sequence identities in the range of ~25–35%. Until recently, among the known lipocalins, rat odorant-binding protein (28) showed the highest sequence identity to aphrodisin (40% at the protein level). However, in the past year, two expressed sequence tag data base entries of 5′-terminal partial cDNAs from mouse have appeared (accession numbers AA172872 and AA172874), which exhibit sequence identities to aphrodisin of >60% at the nucleotide level and ~50% at the amino acid level. Thus, it is presumed that these sequences represent a murine equivalent of aphrodisin. Recently, another nucleotide sequence entry generated by Mameli and co-workers[2] appeared

[2] M. Mameli, D. Pes, I. Andreini, J. Kreiger, H. Breer, and P. Pelosi, unpublished data; EMBL accession no. Y10971 (1997).

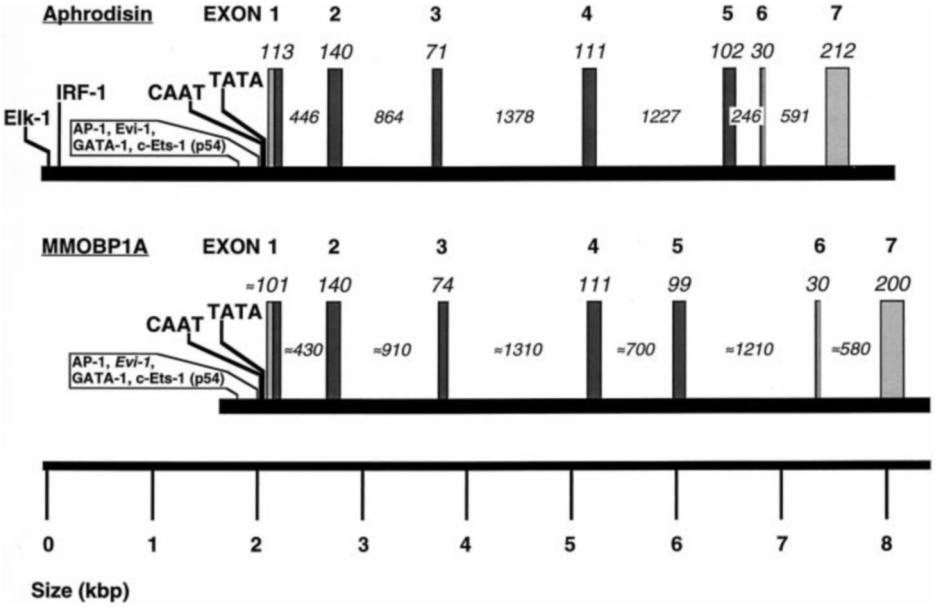

FIG. 1. **Schematic drawings of the structures of the genes for golden hamster aphrodisin and MMOBP1A.** Exon sizes are given in bp above the *boxes* representing the exons. Intron sizes are given in bp between the *boxes*. Translated regions are in *dark gray*, untranslated regions in *light gray*. Transcription factors exhibiting potential binding sites within the gene promoters as well as the TATA and CAAT boxes are indicated. Within the scheme of the MMOBP1A gene, Evi-1 is printed in *italics* because it is the only potential regulating element mentioned with a matrix similarity <0.9.

in the EMBL nucleotide sequence data base. The product of the corresponding gene was designated MMOBP1A. Although the sequence encoding the secretory signal peptide of the precursor was missing, the MMOBP1A cDNA sequence in overlapping regions showed absolute identity to the expressed sequence tag clones mentioned above. Taking together the sequence information of all three clones, a cDNA sequence encoding an entire putative MMOBP1A precursor protein could be assembled. Because of all known lipocalins this protein exhibits the highest sequence identity to aphrodisin at the nucleotide (66%) as well as at the amino acid level (47%), a similar biological function is likely. To obtain further data for the verification of this hypothesis, we decided to compare the structures of the corresponding genes, especially concerning the positions of their introns. For this purpose, we derived MMOBP1A-specific PCR primers (MAP-66–MAP-71) flanking the expected positions of the introns, presuming a distribution nearly identical to that of the aphrodisin gene. At the time we began these experiments, only the expressed sequence tag clones spanning a partial cDNA region including the putative exons 1–4 and the beginning of exon 5 were known. Thus, only primers for the amplification of introns 1–3 could be derived. For amplification of the 5′- and 3′-terminal remnants of the gene, we performed GenomeWalker PCR reactions as described above. From the upstream-directed GenomeWalker PCR we obtained a fragment of ~350 bp including the part of the gene from nucleotide 4 of the translated region up to position −300 relative to the putative cap site. From the downstream-directed GenomeWalker PCR, we obtained a contig of ~4260 bp spanning the region from nucleotide 44 of exon 4 down to ~1400 bp of the 3′-flanking region of the gene. Inner-positioned, PCR-amplified introns 1–3 as well as introns 4–6 included within the downstream GenomeWalker contig were only roughly sequenced to estimate their size. This means that ambiguous nucleotide positions occurring within the sequence that are generated to a particular low amount by the sequencer have not been precisely determined. On the other hand, exons 1–4 were not sequenced but amplified, using the four primer pairs MAPEX-1/2–MAPEX-7/8, and subsequently analyzed on an agarose gel to determine whether they contain additional introns not known from the aphrodisin gene. Indeed, the sizes of the fragments obtained exactly matched those calculated for exons without additional introns (data not shown). Taken together, the amplification and sequencing of the MMOBP1A gene fragments and comparison with the golden hamster aphrodisin gene revealed an almost identical positioning of the introns (Figs. 1 and 2), verifying the relation of both genes to one another. However, only introns 1–3 and 6 match the sizes of the corresponding aphrodisin gene introns, because introns 4 and 5 show deviations (Fig. 1).

Analysis of the Aphrodisin gene Promoter and Sequence Comparison with the MMOBP1A Promoter—The promoter region of the aphrodisin gene has already been determined by primer extension analysis (21). To verify its functionality *in vivo*, we performed several promoter-luciferase reporter gene assays with different subfragments of the promoter and a selected number of cell lines. For this purpose, a 2144-bp aphrodisin gene 5′-flanking fragment was amplified by means of the primer OZ-1, derived from the upstream terminus of the 5.- kbp *Sst*I fragment, and the second primer OZ-2, spanning the region from the adenosine nucleotide of the ATG translational start codon up to the position located 23 nucleotides upstream of the ATG start codon and 26 nucleotides downstream from the already determined cap site. The fragment obtained was cloned into the luciferase reporter gene vector pGL2 basic. Two additional clones, containing 1170- and 669-bp subfragments of the 2144-bp fragment, were generated as described above.

Because no cell line that seemed to be ideal for aphrodisin gene promoter analysis (for instance, a hamster vaginal epithelium cell line) was available, we tested the cell lines RK 13 (rat kidney), CRL 6176 (cat salivary glands), and T 84 (human colon carcinoma) for their suitability for aphrodisin gene promoter activity studies. Of these cell lines, only the human T 84 cells gave reproducible results and showed a significant promoter functionality. The results obtained are presented in Fig. 3: the entire aphrodisin gene 5′-flanking fragment of 2144 bp exhibited a promoter activity that is 36-fold above the activity of the basic vector, whereas the 1170- and 669-bp fragments showed 17- and 16-fold activities, respectively. These results strongly indicate the functionality of the aphrodisin gene promoter region in T 84 cells. They further indicate the existence of an activating element within the 1-kbp region, starting ~1150 bp upstream of the cap site.

As another strategy to identify potential regulatory regions within the aphrodisin gene and its 5′-flanking region, we analyzed its nucleotide sequence using the MatInspector program

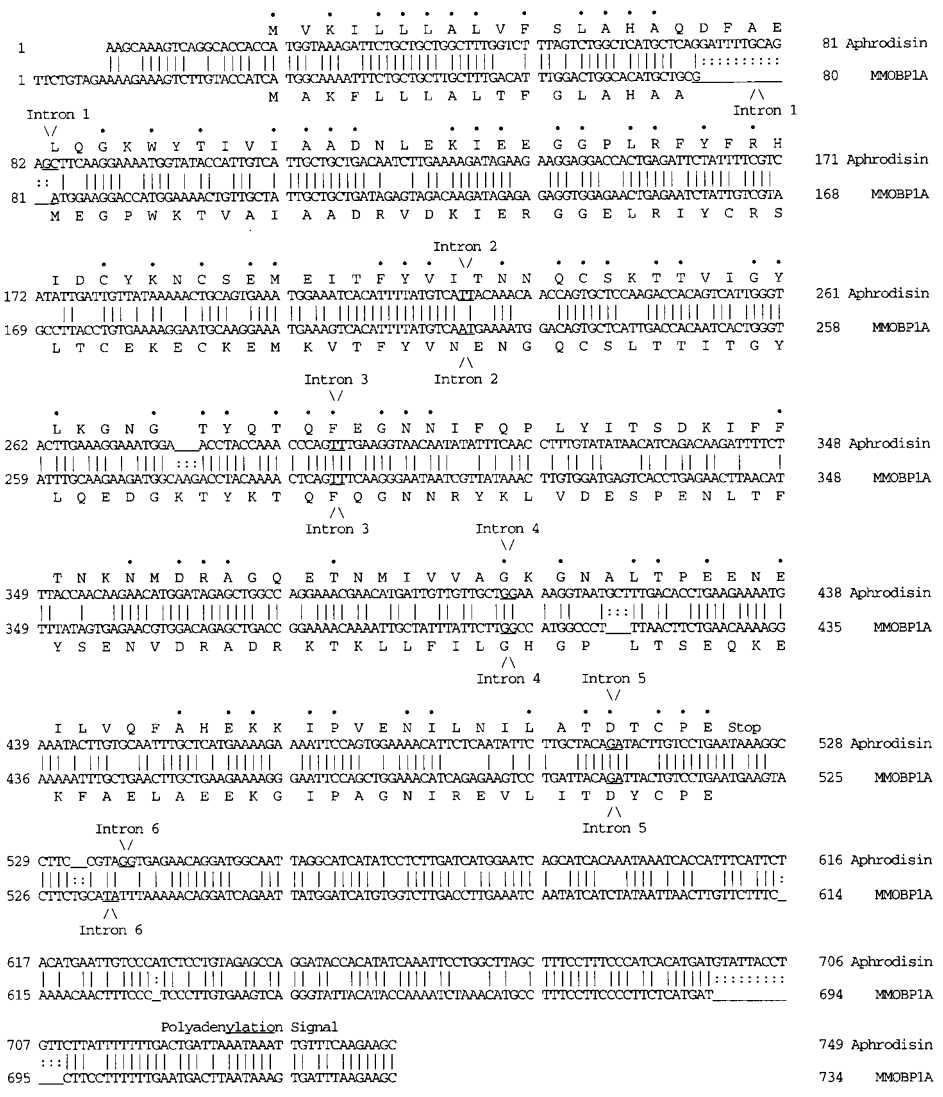

FIG. 2. **Comparison of the cDNA and amino acid sequences of hamster aphrodisin and MMOBP1A.** Nucleotides identical in both cDNAs are marked with *vertical dashes*; agreeing amino acids are marked with *black dots* above the aphrodisin sequence. The positions of the introns of both genes are indicated; the nucleotides flanking the introns are *underlined*.

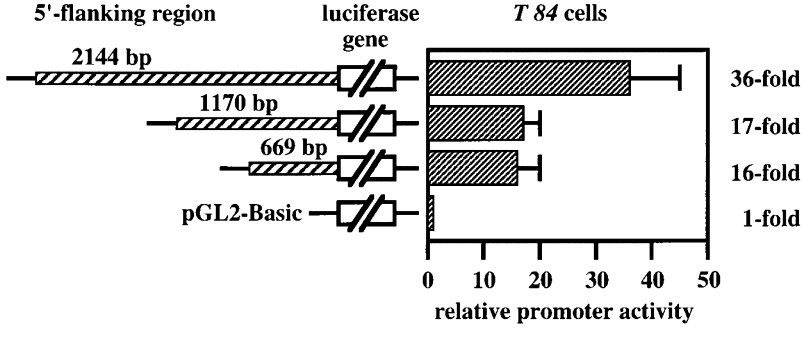

FIG. 3. **Results of the luciferase reporter gene assays with three different aphrodisin promoter fragments in T 84 cells.** The relative promoter activities were calculated from three independent assays.

(23). The 300-bp 5′-flanking region of the MMOBP1A gene obtained by the above-mentioned genome walking PCR was sequenced precisely on both strands and was analyzed in the same way as the aphrodisin gene promoter. For detection of high probability regulatory elements, the core similarities generally had to be 100%, whereas the matrix similarities had to be at least 90%. In addition, two alternative criteria had to be fulfilled. First, the core sequence of a regulatory element had to occur in the promoter regions of both genes, the golden hamster aphrodisin gene and the MMOBP1A gene. Second, if the first requirement could not be met, the random expectation value for a high probability regulating element within the aphrodisin gene promoter had to be 0.1 at the most.

Using this strategy, potential binding sides for the following transcription factors could be detected within the promoters of both genes: AP-1 (29), Evi-1 (30), GATA-1 (twice) (31), and c-Ets-1(p54) (twice) (32). Furthermore, CAAT boxes in reverse complementary orientation and TATA boxes occur in the expected positions. In addition, putative binding sites for Elk-1 (33, 34) and IRF-1 (35, 36) are represented within the aphrodisin gene 5′-flanking region (Figs. 1 and 4 and Table I).

Expression of the Aphrodisin Gene—The expression of the

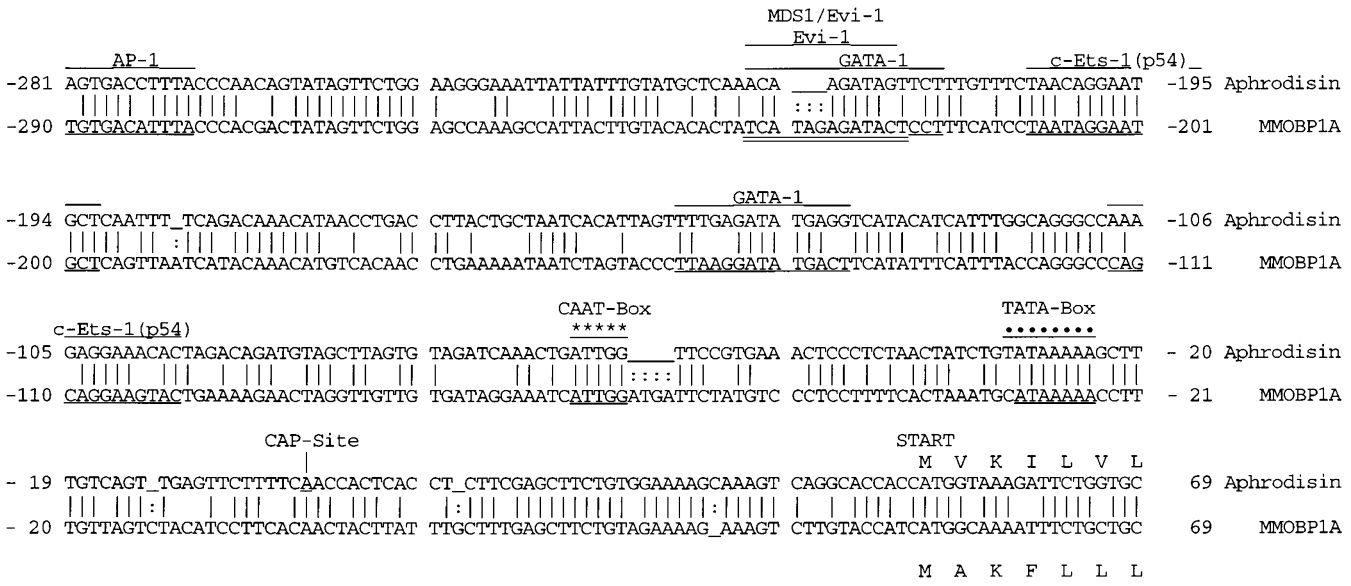

FIG. 4. **Comparison of two corresponding parts of the golden hamster aphrodisin and MMOBP1A gene promoters.** The enumeration of the nucleotides is relative to the cap site and in the case of MMOBP1A to the putative cap site. Potential regulatory elements are indicated (also see Table I). The similarity of the presented regions is ~67%.

TABLE I

Potential regulatory elements of the aphrodisin and MMOBP1A gene promoters identified by the MatInspector program

The core similarity of all elements had to be 1.0. With the exception of the MMOBP1A Evi-1 site, the matrix similarity had to be at least 0.9. Core sequences are in capital letters, matrix sequences in small letters. The IRF-1 site (printed in italics) occurs on the minus strand, all other elements on the plus strand. The positions are relative to the cap site and in the case of MMOBP1A to the putative cap site (see Fig. 4). NA, not applicable.

Factor, element	Sequence	Position	Matrix similarity	Random expectation	Gene
Elk-1	GGAA	−2081	NA	0.06	Aphrodisin
IRF-1	*gaaaaatGAAAaa*	−1972	0.915	0.02	Aphrodisin
AP-1	agTGACccttta	−281	0.916	2.45	Aphrodisin
	tgTGACattta	−290	0.901		MMOBP1A
Evi-1	acaAGATag	−224	0.961	0.02	Aphrodisin
MDS1/Evi-1	tagAGATac	−230	0.603		MMOBP1A
GATA-1	aacaaGATAgttct	−225	0.930	2.27	Aphrodisin
	atagaGATActcct	−231	0.927		MMOBP1A
	tttgaGATAtgagg	−144	0.947		Aphrodisin
	ttaagGATAtgact	−149	0.928		MMOBP1A
c-Ets-1 (p54)	taacaGGAAtgct	−204	0.932	7.63	Aphrodisin
	taataGGAAtgct	−210	0.908		MMOBP1A
	aaagaGGAAacac	−108	0.941		Aphrodisin
	cagcaGGAAgtac	−113	0.965		MMOBP1A

aphrodisin gene in different segments of the female hamster genital tract has already been demonstrated. Because aphrodisin probably functions as a transporter for low molecular mass hydrophobic pheromone molecules, which act via the vomeronasal organ of the male hamster, additional expression of the gene in several regions of the hamster throat seemed to be likely. We therefore performed Western blot analysis with protein extracts (6 μg of total protein) from the saliva of male and female golden hamsters. Surprisingly, and contrary to our expectations, we obtained signals in the expected size range only from female animals (Fig. 5*A*). In the saliva of male golden hamsters, which are the recipients of pheromonal signals from the female animals, no aphrodisin-specific signals were detectable. To verify these results at the nucleic acid level, we also performed RT-PCR analysis with total RNA extracted from the parotid glands of female and male hamsters and, as a positive control, from hamster vagina. In this case, we also obtained significant signals only from female hamster parotid glands (Fig. 5*B*), verifying the results of the Western blot analysis. The corresponding PCR fragments have been reamplified, directly sequenced, and identified as aphrodisin-specific. Unexpectedly, the data obtained clearly indicate an expression of the aphrodisin gene in female golden hamster parotid glands and the secretion of the protein into the female hamster's saliva.

Another question arising in the context of aphrodisin as a putative pheromone transporter concerns the dependence of gene expression on the stage of fertility of juvenile female hamsters. Provided that aphrodisin functions exclusively as a transporter for sex pheromones, its secretion into female hamster vaginal discharge only would make sense from the time the newborn hamsters reach fertility. To test this hypothesis, we performed Western blot analysis on vaginal discharge from two female animals taken from day 22 to day 43 after birth. Golden hamsters normally reach fertility between days 28 and 40 after birth, whereby both values represent the lowest and highest values, respectively, as known from the literature (37). A comparable high concentration of aphrodisin in vaginal discharge was detectable within all of the analyzed samples (Fig. 6). Thus, secretion of aphrodisin into vaginal discharge does not seem to depend on the point at which female animals reach fertility.

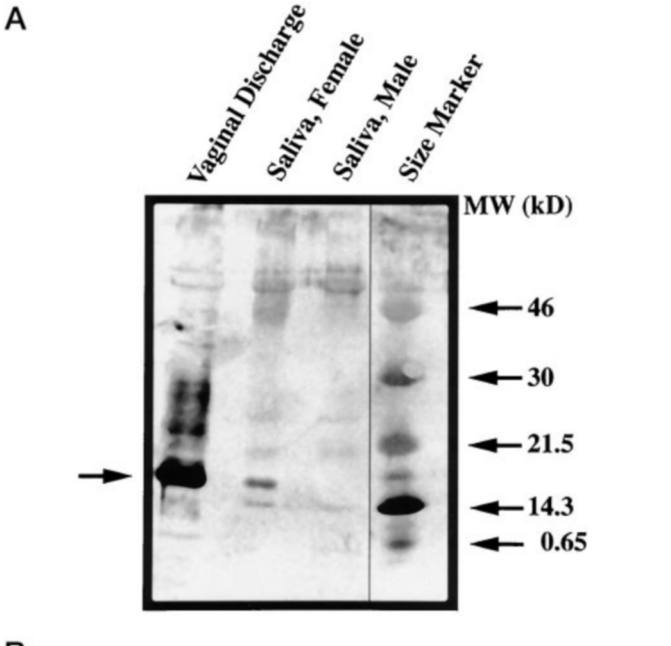

FIG. 5. **Detection of aphrodisin gene expression in female golden hamster parotid glands.** *A*, Western blot with saliva from golden hamsters of both sexes and vaginal discharge. The aphrodisin-specific signal appearing in the expected size range is indicated by an *arrow*. Aphrodisin immunoreactivity is detectable in vaginal discharge and saliva of female hamsters. Saliva of male animals does not show any aphrodisin immunoreactivity. *B*, aphrodisin-specific RT-PCR analysis of cDNA from golden hamster uterus (positive control), parotid glands from female and male golden hamsters and rabbit uterus (negative control). Shown is the autoradiograph of a Southern blot with reaction products of an aphrodisin-specific RT-PCR. The blot was hybridized with an oligonucleotide probe, the sequence of which is positioned between the two PCR primers relative to the sequence of the aphrodisin cDNA. The *number* of cycles is indicated above the autoradiograph. Positive signals are visible from the uterus and female hamster parotid gland. Male hamster parotid gland and rabbit uterus (negative control) do not show any signal.

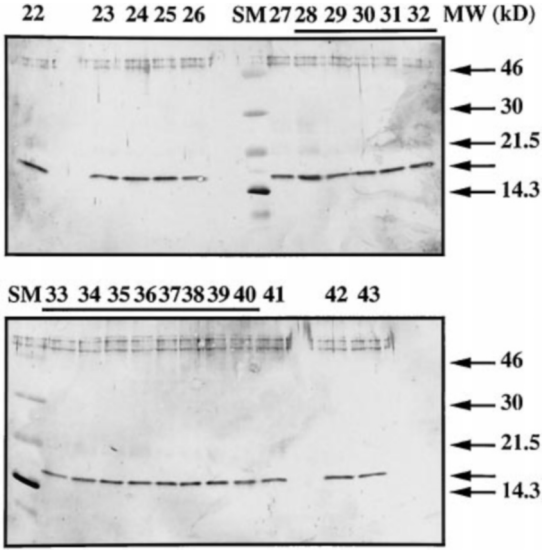

FIG. 6. **Western blots with samples of vaginal discharge from a female hamster taken from days 22–43 after birth.** The *number* of the day at which the sample was taken is indicated above the blots. The time period when hamsters normally reach fertility (days 28–40) is marked by a *horizontal line*. Aphrodisin immunoreactivity in the expected size range is visible in all samples, even already at day 22 after birth.

DISCUSSION

One of the main reasons for our decision to characterize the aphrodisin cDNA and gene was the intention to identify and investigate related systems in species other than the hamster. Indeed, sequence comparisons with data base entries led to the discovery of the aphrodisin-related MMOBP1A, the gene structure of which is reported in this paper. As in the case of the aphrodisin gene, it exhibits a seven-exon/six-intron structure. The construction of a phylogenetic tree of several lipocalins based on their amino acid sequences was already performed by Igarashi and co-workers (38). As an alternative, we compared the aphrodisin gene with other lipocalin genes (MMOBP1A, α2UG, βLG, PP14, and RPDS; see Ref. 38) also possessing seven exons under the criterion of their structure. For this purpose, we added the absolute values of size deviations in base pairs of each exon of a given gene from the corresponding exon of the aphrodisin gene. The resulting value was expected to be low for closely related genes and high for genes showing a more distant relation to the aphrodisin gene. Using this strategy, the following relation was obtained in descending order: MMOBP1A > PP14 > βLG > α2UG > RPDS. Despite the fact that, at this time, the structure of the MMOBP1A gene was still not known, the order of relation obtained fully verifies the phylogenetic tree as presented by Igarashi and co-workers (38). Thus, the close relation of MMOBP1A to aphrodisin is demonstrated at the level of sequence identity, as well as at the level of gene structure.

The 290-bp region following the putative cap site of the MMOBP1A gene in the 5′ direction also shows a striking sequence identity of 67% to the corresponding region of the aphrodisin gene. This similarity is additionally reflected in the pattern of potential regulatory elements in both gene promoters. As described above, we have identified potential binding sites for the transcription factors Elk-1, IRF-1, AP-1, Evi-1, GATA-1 (twice), and c-Ets-1 (twice) within the 2144-bp 5′-flanking region of the aphrodisin gene (Fig. 4). In the case of the MMOBP1A gene, only a 290-bp fragment of the 5′-flanking region was available. However, within this fragment the putative binding sites for AP-1, Evi-1, GATA-1 (twice), and c-Ets-1 (p54) (twice) as well as the putative TATA and CAAT boxes occur at positions identical to those within the aphrodisin gene. In addition, as in the aphrodisin gene promoter the CAAT element of the MMOBP1A gene exhibits a reverse complementary orientation. These findings indicate the significance of at least a part of the identified elements and comparable mechanisms of regulation of both genes.

Among the corresponding transcription factors possibly interacting with the aphrodisin gene promoter, Evi-1 is the most probable candidate. It functions as a transcriptional repressor (39) but also occurs in an N-terminally elongated form, MDS1/Evi-1, which exhibits a strong activating effect (40). Both forms recognize the same motif containing an AGAT core sequence (see Table I). In mice, besides being found in a few other organs, an Evi-1 gene-specific mRNA was already localized within the uterus and during embryogenesis within regions of that part of

the nasal cavity that later forms the vomeronasal organ (41). We have already demonstrated the expression of the aphrodisin gene in the golden hamster uterus (22). On the other hand, the synthesis of the putative pheromone transporter aphrodisin within the vomeronasal organ would also make sense, because this organ is responsible for pheromone detection. Thus, an involvement of Evi-1 or MDS1/Evi-1 in aphrodisin gene regulation seems to be likely. Further experiments for the verification of this presumption are planned.

As presented in this paper, we have performed initial luciferase reporter gene assays to test the functionality of the aphrodisin gene promoter *in vivo*. Although we could show a significant promoter activity in T 84 cells, this cell system does not seem to be ideal for aphrodisin promoter studies, and the measured maximum relative activity of 36-fold is too low to correlate with the extremely high level of aphrodisin gene expression observed in the female golden hamster genital tract (21, 22). However, the fact that the largest tested promoter fragment of 2144 bp containing the potential binding sites for Elk-1 and IRF-1 shows a significant 2-fold enhancement of promoter activity compared with the 1170-bp promoter fragment might indicate the actual involvement of at least one of these transcription factors in aphrodisin gene regulation.

Performing RT-PCR and Western blot analysis, we succeeded in detecting the parotid glands of female golden hamsters as hitherto unknown loci of aphrodisin gene expression. The hypothesis that led us to investigate these organs stands in contradiction to the results obtained. We first expected an involvement of endogenously synthesized aphrodisin in the transport of a copulatory behavior-stimulating pheromone of the female hamster to the vomeronasal organ of the male hamster. Parotid glands were chosen as objects of investigation because they are located within the hamster's throat cavity and are relatively easy to isolate. The fact that not the male hamsters, as pheromone recipients, but the female hamsters express the aphrodisin gene within their parotid glands may lead to the discovery of as yet unknown aphrodisin functions. For instance, female hamsters lick their offspring in an intensive way, thereby probably transferring saliva-borne aphrodisin onto its skin. Thus, aphrodisin-mediated effects on the social behavior of hamsters that are possibly important for the protection of young animals are thinkable. The second unexpected result of our investigations, namely the abundant occurrence of aphrodisin in vaginal discharge before the female animals reach fertility, would be in agreement with this hypothesis. On the other hand, in young nonfertile female hamsters, the synthesis of aphrodisin as an apoprotein lacking its putative ligand required for the pheromonal effect must also be taken into consideration. Possibly, this ligand is synthesized from the time when female animals reach fertility, then binding to the already present aphrodisin to generate the bioactive complex. Because several lipocalins are capable of binding different ligands, a variable modulation of the aphrodisin-ligand complex function by ligands specific for a certain tissue or developmental stage also cannot be excluded.

To unambiguously clarify these questions, further studies on the aphrodisin-dependent social and copulatory behavior of live animals have to be performed. A great step toward this goal is given by the fact that an aphrodisin-related system of the mouse has now been identified, enabling the construction and analysis of MMOBP1A knock-out mice. Furthermore, it is now possible to construct hybridization probes and primers corresponding to regions highly conserved within the genes for aphrodisin and MMOBP1A, which may be used for the detection of related systems in other mammals. Although the importance of aphrodisin and related lipocalins for the reproduction of mammals at this time is difficult to predict, future use of such proteins in the breeding of animals is thinkable.

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REFERENCES

1. Flower, D. R. (1996) *Biochem. J.* **318,** 1–14
2. Urade, Y., Nagata, A., Suzuki, Y., Fujii, Y., and Hayaishi, O. (1989) *J. Biol. Chem.* **264,** 1041–1045
3. Nagata, A., Suzuki, Y., Igarashi, M., Eguchi, N., Toh, H., Urade, Y., and Hayaishi, O. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 4020–4024
4. Schmale, H., Holtgreve-Grez, H., and Christiansen, H. (1990) *Nature* **343,** 366–369
5. Bläker, M., Kock, K., Ahlers, C., Buck, F., and Schmale, H. (1993) *Biochim. Biophys. Acta* **1172,** 131–137
6. Morrow, D. M., Xiong, N., Getty, R. R., Ratajczak, M. Z., Morgan, D., Seppala, M., Riittinen, L., Gerwitz, A. M., and Tykocinski, M. L. (1994) *Am. J. Pathol.* **145,** 1485–1495
7. Boyles, J. K., Notterpek, L. M., and Anderson, L. J. (1990) *J. Biol. Chem.* **265,** 17805–17815
8. Spreyer, P., Schaal, H., Kuhn, G., Rothe, T., Unterbeck, A., Olek, K., and Müller, H. W. (1990) *EMBO J.* **9,** 2479–2484
9. Flower, D. R. (1994) *FEBS Lett.* **354,** 7–11
10. Riley, C. T., Barbeau, B. K., Keim, P. S., Kézdy, F. J., Heinrikson, R. L., and Law, J. H. (1984) *J. Biol. Chem.* **259,** 13159–13165
11. Bishop, R. E., Penfold, S. S., Frost, L. S., Höltje, J.-V., and Weiner, J. H. (1995) *J. Biol. Chem.* **270,** 23097–23103
12. Flower, D. R., Sansom, C. E., Beck, M. E., and Attwood, T. K. (1995) *Trends Biochem. Sci.* **20,** 498–499
13. Singer, A. G., Macrides, F., Clancy, A. N., and Agosta, W. C. (1986) *J. Biol. Chem.* **261,** 13323–13326
14. Henzel, W. J., Rodriguez, H., Singer, A. G., Stults, J. T., Macrides, F., Agosta, W. C., and Niall, H. (1988) *J. Biol. Chem.* **263,** 16682–16687
15. Clancy, A. N., Macrides, F., Singer, A. G., and Agosta, W. C. (1984) *Physiol. Behav.* **33,** 653–660
16. Moran, D. T., Jafek, B. W., and Rowley, J. C. (1991) *J. Steroid Biochem. Mol. Biol.* **39,** 545–552
17. Steensas, L. J., Lavker, R. M., Monti-Bloch, L., Grosser, B. I., and Berliner, D. L. (1991) *J. Steroid Biochem. Mol. Biol.* **39,** 553–560
18. Monti-Bloch, L., and Grosser, B. I. (1991) *J. Steroid Biochem. Mol. Biol.* **39,** 573–582
19. Singer, A. G., and Macrides, F. (1990) *Chem. Senses* **15,** 199–203
20. Kroner, C., Breer, H., Singer, A. G., and O'Connell, R. J. (1996) *NeuroReport* **7,** 2989–2992
21. Mägert, H. J., Hadrys, T., Cieslak, A., Gröger, A., Feller, S., and Forssmann, W. G. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 2091–2095
22. Kruhøffer, M., Bub, A., Cieslak, A., Adermann, K., Kunstyr, I., Forssmann, W. G., and Mägert, H. J. (1997) *Cell Tissue Res.* **287,** 153–160
23. Quandt, K., Frech, K., Karas, H., Wingender, E., and Werner, T. (1995) *Nucleic Acids Res.* **23,** 4878–4884
24. Pardigol, A., Mägert, H. J., Hill, O., and Forssmann, W. G. (1996) *Biochem. Biophys. Res. Commun.* **224,** 638–644
25. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Ehrlich, H. A. (1988) *Science* **239,** 487–491
26. Siebert, P. D., Chenchik, A., Kellog, D. E., Lukyanov, K. A., and Lukyanov, S. A. (1995) *Nucleic Acids Res.* **23,** 1087–1088
27. Mount, S. M. (1982) *Nucleic Acids Res.* **10,** 459–472
28. Pevsner, J., Reed, R. R., Feinstein, P. G., and Snyder, S. H. (1988) *Science* **241,** 336–339
29. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, C., Herrlich, P., and Karin, M. (1987) *Cell* **49,** 729–739
30. Perkins, A. S., Fishel, R., Jenkins, N. A., and Copeland, N. G. (1991) *Mol. Cell. Biol.* **11,** 2665–2674
31. Evans, T., Reitman, M., and Felsenfeld, G. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 5976–5980
32. Ho, I. C., Bhat, N., Gottschalk, L. R., Lindenstein, T., Thompson, C. B., Papas, T. S., and Leiden, J. M. (1990) *Science* **250,** 814–818
33. Hipskind, R. A., Rao, V. N., Moeller, C. G. F., Reddy, E. S. P., and Nordheim, A. (1991) *Nature* **354,** 531–534
34. Rao, V. N., and Reddy, E. S. P. (1992) *Oncogene* **7,** 65–70
35. Fujita, T., Sakakibara, J., Yoshiaki, S., Miyamoto, M., Kimura, Y., and Taniguchi, T. (1988) *EMBO J.* **7,** 3397–3405
36. Kessler, D. S., Levy, D. E., and Darnell, J. E., Jr. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 8521–8525
37. Güttner, J., Bruhin, H., and Heinecke, H. (eds) (1993) *Wörterbuch der Versuchstierkunde*, Gustav Fischer, Jena, Stuttgart
38. Igarashi, M., Nagata, A., Toh, H., Urade, Y., and Hayaishi, O. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 5376–5380
39. Bartholomew, C., Kilbey, A., Clark, A. M., and Walker, M. (1997) *Oncogene* **14,** 569–577
40. Soderholm, J., Kobayashi, H., Mathieu, C., Rowley, J. D., and Nucifora, G. (1997) *Leukemia* **11,** 352–358
41. Perkins, A. S., Mercer, J. A., Jenkins, N. A., and Copeland, N. G. (1991) *Development* **111,** 479–487