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A Relative of the Catalytic Subunit of Cyclic AMP-Dependent Protein Kinase in *Aplysia* Spermatozoa

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Transcripts encoding C_{APL-B}, an apparent member of the cyclic-nucleotide-regulated kinase subfamily in *Aplysia californica*, are found exclusively in the ovotestis and are concentrated in meiotic and postmeiotic spermatogenic cells. The C_{APL-B} polypeptide is present in mature spermatozoa, suggesting that the kinase plays a part in regulating events associated with fertilization.

One branch of the protein kinase (PK) superfamily consists of closely related Ser/Thr kinases regulated by the second messengers cyclic AMP (cAMP), cGMP, and Ca^{2+}-diacylglycerol (the AGC kinases [8, 12, 14]). We have been examining the isoforms of cAMP-dependent protein kinase (PKA) in the nervous system of the marine mollusk *Aplysia californica* (3). During the course of this work, a new kinase related to the AGC kinases, called sak (spermatozoon-associated kinase), has been identified, the *Aplysia* form of which is C_{APL-B}.

A genomic λ phage library was screened at very low stringency (3, 19) by using two nonoverlapping mouse C_α cDNA probes (MC4 and MC1A [30]). Sequence from clone V79-8 (Fig. 1a) revealed an exon encoding part of a previously undescribed kinase designated C_{APL-B}. To obtain a probe to screen a cDNA library for C_{APL-B} and to determine the tissue from which the library should be constructed, a reverse transcriptase-polymerase chain reaction (RT-PCR) experiment was conducted with a 3′ antisense primer identical to a sequence in C_{APL-A} (*Aplysia* PKA) that is highly conserved in all cyclic-nucleotide-dependent kinases (12) and with a 5′ sense primer identical to part of the C_{APL-B} genomic sequence and poorly conserved in other kinases (Fig. 1a). The predicted size of the PCR product was 282 bp (12). A DNA of this size, which hybridized to a C_{APL-B}-specific oligonucleotide probe, was derived from ovotestis RNA but not from RNA from the central nervous system or buccal muscle (Fig. 1b). The presence of the C_{APL-B} transcript in the ovotestis was confirmed by Northern (RNA) analysis (Fig. 1c). On the basis of these findings, a λ gt10 cDNA library was made by random priming of adult *Aplysia* ovotestis poly(A)$^+$ RNA and screened with a probe generated from the PCR product. The sequence of the insert from one of several positive phages, SB-OT-18, revealed an open reading frame of 351 amino acids (40,947 Da; Fig. 2).

Cursory examination of the C_{APL-B} sequence placed it in the subfamily containing PKA, cGMP-dependent protein kinase (PKG), and protein kinase C (PKC) (AGC subfamily). Therefore, a more detailed comparison was made with these kinases by aligning the catalytic domains (Fig. 3A) as defined by Hanks et al. (12). The sequence identities of C_{APL-B} with close relatives were as follows: PKA catalytic subunits (human C_α, C_β, and C_γ and *Aplysia* C_{APL-A1} and C_{APL-A2}), 55 to 57%; bovine PKG, 43%; bovine PKCα, 36%; and rabbit nPKCε, 35%. C_{APL-B} does not correspond to either of the two *Drosophila* PKA relatives, DC1 and DC2 (16). On the basis of this information, C_{APL-B} can be regarded as a distinct member of the cyclic-nucleotide-dependent protein kinase subfamily (note that isoforms such as the mammalian PKA C_α, C_β, and C_γ subunits [2] have far higher sequence identity, e.g., human α and β, 95%; α and γ, 87%; and β and γ, 83%). Alternatively, C_{APL-B} can be viewed as a member of the AGC subfamily, which in addition to the cyclic-nucleotide-dependent protein kinases includes Ca^{2+}-dependent PKC (PKCα, -βI, -βII, and -γ) and the recently identified Ca^{2+}-independent PKC (nPKCε [1, 20, 21]). C_{APL-B} does not possess a fused regulatory domain such as that in PKG or PKC. The absence of a Gly residue immediately after the initiator Met indicates that, unlike the C subunit of PKA (5), the C_{APL-B} polypeptide is unlikely to be myristylated (29). The relationships between the AGC kinases are clarified in the limbs of the phylogenetic tree shown in Fig. 3b.

To ascertain whether C_{APL-B} was expressed only in cells derived from the ovotestis, an RT-PCR experiment was carried out on RNA isolated from an adult animal from which the reproductive tract had been removed. The experiment confirmed that no other tissues contained C_{APL-B} transcripts (Fig. 4a). Similar experiments were then performed on immature animals, which by the criteria of Kriegstein (17) and Carew (4) were in late stage 12. At this point in development, the animals possess most of the physical and behavioral attributes of the adult except for a mature reproductive system (4, 17). C_{APL-B} transcripts were absent in small animals and present in increasing amounts in larger specimens (Fig. 4a).

A. californica is a non-self-fertilizing hermaphrodite. The gonad, or ovotestis, contains both oocytes and spermatozoa and their progenitor cells. The location of the transcripts within the adult ovotestis was examined by in situ hybridization (Fig. 4b). Transcripts were not detected in oocytes at any stage of maturation. The highest concentrations were observed in spermatogenic cells, with the first appearance

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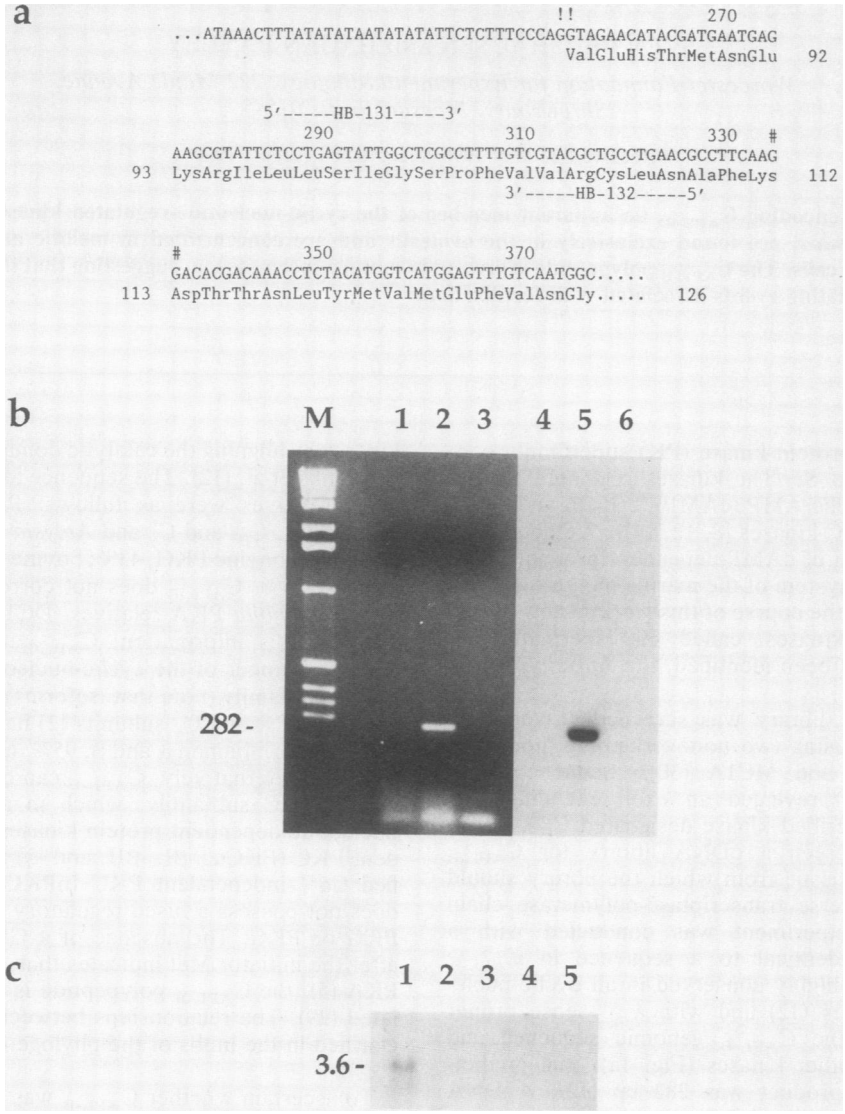

FIG. 1. An *Aplysia* gene encoding a protein kinase related to PKA is transcribed in the ovotestis. (a) Partial sequence of an exon in *Aplysia* genomic clone V79-8, showing the positions of the intron-exon boundary (!!) (which has no equivalent in the mouse C_α gene [6]), the exon 4-5 boundary from the mouse C_α gene (##) (6), the sense PCR primer HB-131, and the oligonucleotide probe HB-132. The numbering is that of the full cDNA sequence (Fig. 2). (b) RT-PCR amplification of $C_{APL\text{-}B}$ transcripts. Total RNA samples from the *Aplysia* nervous system (lanes 1 and 4), ovotestis (lanes 2 and 5), and buccal muscle (lanes 3 and 6) were subjected to RT-PCR amplification using the 3′ primer HB-134 and the 5′ primer HB-131 (see panel a and Fig. 2 for the locations and sequences of the primers). The amplification products were separated by agarose gel electrophoresis and stained with ethidium bromide (lanes 1 to 3) or transferred to nylon and probed with a 5′-end-labeled internal oligonucleotide, HB-132 (lanes 4 to 6). The three lower bands of the molecular size markers (lane M) are 396, 344, and 298 bp. Given the conserved structure of protein kinases (12) and the choice of primers and probe, an amplification product of 282 bp was predicted from samples in which $C_{APL\text{-}B}$ transcripts were present, while related $C_{APL\text{-}A}$ transcripts would not be detected. The PCR product was sequenced directly and found to include the genomic sequence reported in panel a. (c) Northern blot of *Aplysia* RNAs. A poly(A)$^+$ RNA of 3.6 kb in ovotestis (lane 1) hybridized to a $C_{APL\text{-}B}$ probe. The following other RNAs did not hybridize: lane 2, ovotestis, poly(A)$^-$; lane 3, heart, poly(A)$^+$; lane 4, buccal muscle, poly(A)$^+$; lane 5, central nervous system (pooled major ganglia), poly(A)$^+$. The probe was transcribed with Klenow fragment from the $C_{APL\text{-}B}$ PCR product by using the primer HB-134. The final wash was in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate at 65°C before exposure to X-ray film for 3 days.

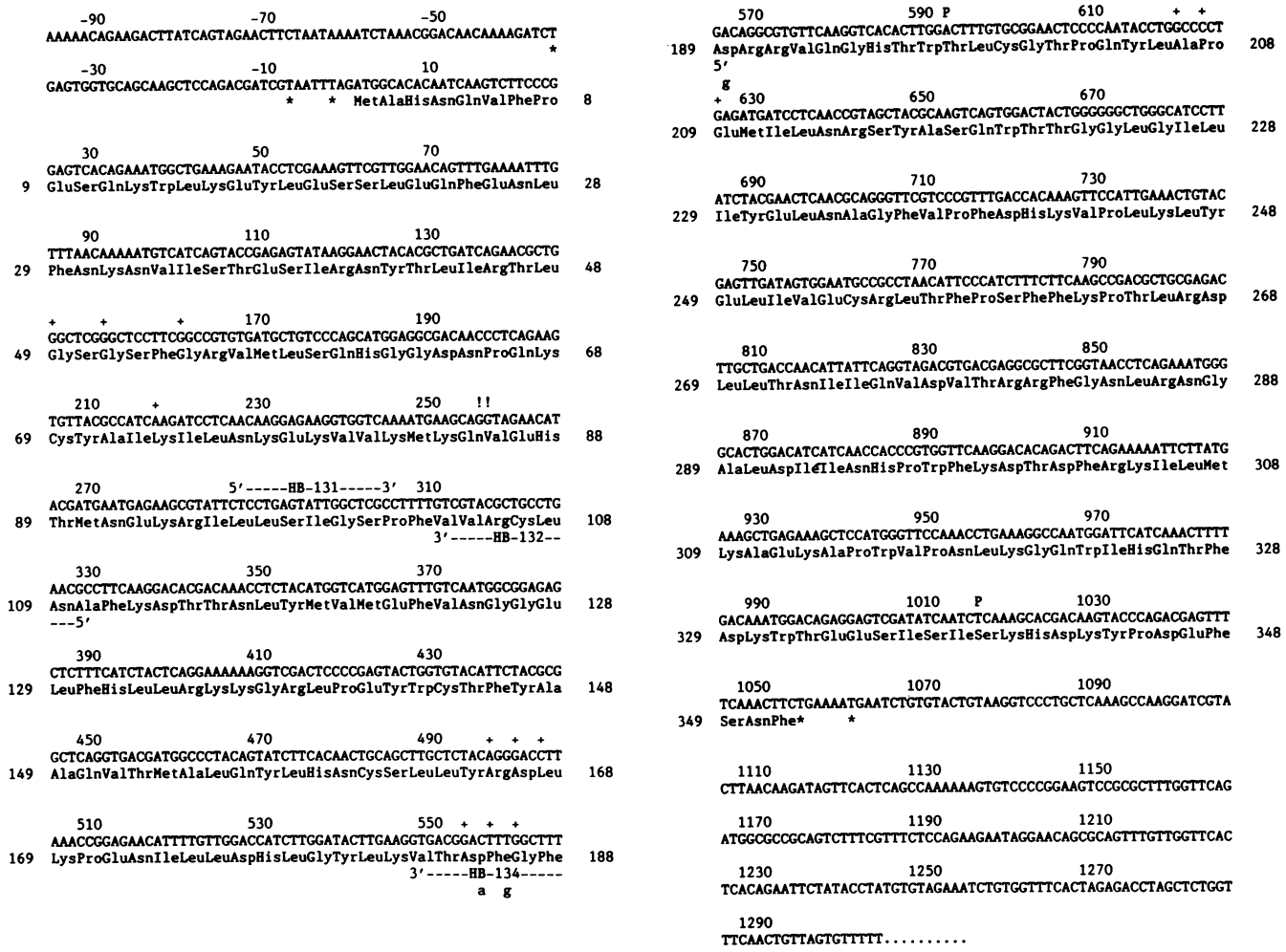

FIG. 2. Sequence of C_{APL-B} cDNA and its translation product. The designated initiation codon is preceded by stop codons (*) in all three reading frames. Also indicated are the exon-exon boundary (!!) deduced from the genomic sequence in Fig. 1a, short sequences conserved in all protein kinases (+) (12, 14), sites that are phosphorylated in the homologous PKA C subunit (P) (26), and the oligonucleotides used in the experiments whose results are shown in Fig. 1b and 4a (HB-131 [sense PCR], HB-132 [C_{APL-B} probe], and HB-134 [antisense PCR; mismatches shown in lowercase]). Following the preparation of nested deletions as described by Henikoff (13), both strands of a 1.5-kb cDNA clone, SB-OT-18, were sequenced by using miniprep DNA as the template. The untranslated sequence upstream from residue −33 was obtained from a separate overlapping cDNA, SB-OT-11.

in meiotic spermatocytes. C_{APL-B} transcripts remained at the postmeiotic small round spermatid stage but were absent in elongating spermatids. Mature sperm cells are incapable of both transcription and protein synthesis, and polypeptides required by them must therefore be made during spermatogenesis. To determine whether the C_{APL-B} transcripts seen in spermatocytes are indeed translated and to ascertain whether the translation products are retained in mature spermatozoa, an immunoblotting experiment was performed by using an affinity-purified antipeptide antibody (OK-C1) directed against the carboxyl terminus of the polypeptide (Fig. 4c). An immunoreactive polypeptide of ~40,000 Da was detected in homogenates of sperm cells (Fig. 4c, lane 3).

Although the experiments described above do not rule out the existence of further sak isoforms in either germ cells or somatic cells, the distribution of C_{APL-B} transcripts is highly tissue specific. In contrast, transcripts encoding the two isoforms of the catalytic subunit of *Aplysia* PKA have been detected in all tissues examined so far (3). During spermatogenesis several Ser/Thr and Tyr kinase mRNAs are differentially expressed (2, 18, 22, 25). Other than C_{APL-B}, three kinases expressed solely in male germ cells have previously been identified in mammals: PSK-C3, a relative of the catalytic subunit of phosphorylase *b* kinase (11); C_γ, an isoform of the catalytic subunit of PKA (2); and a relative of the cdc2 kinase, mak (18). Selective gene transcription in spermatogenic cells has long been recognized and often begins during meiosis, which is the case with C_{APL-B} (31). For example, in trout, protamine mRNA is made in spermatocytes, stored, and later translated at the spermatid stage (15). Transcription in haploid spermatids has also been detected (7, 23), and because C_{APL-B} transcripts remain relatively abundant in early haploid cells, we cannot rule out renewed transcription at this stage.

a

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          1                                                  50
bovPKA    DQFER K   T        VK M.ETGNH.  M   D Q    L  I
aplPKA-1  DDFDR K   T        VG K ESRNF.  M   D Q    L
aplPKA-2  DDFDR K   T        VG K ESRNF.  M   D Q    L
aplSAK    RNYTLIRTLGSGSFGRVMLSQHGGDNPQKCYAIKILNKEKVVKMKQVEHT
bovPKG    SDFNI D   V G    E V LKSEE.SKTF M   K RHI DTR Q  I
bovPKC    TDFNFLMV  K    K   ADRK TEEL..      K DV IQDDD  CT
rabNPKC   DEFNF KV  K    K   AELK KDEV..  V V K DVILQDDD DCT

          51                                                 100
bovPKA    L      QAV.NF  L KLEFS   NS       Y P   M SH  RI
aplPKA-1  L   K  Q  .NF  L KLEYS   NS     L   T   M SH  RI
aplPKA-2  L   K  Q  .NF  L KLEYS   NS     L   T   M SH  RI
aplSAK    MNEKRILLSI.GSPFVVRCLNAFKDTTNLYMVMEFVNGGELFHLLRKKGR
bovPKG    RS  Q MQGA.H D I  LYRT   SKY   L  ACL    WTI  DR S
bovPKC     V   V ALLDKP  LTQLHSC QTVDR  F   Y    D MYHIQQV K
rabNPKC    T     ALARKH YLTQLYCC QTKDR FF   Y    D MFQIQRSRK

          101                                                150
bovPKA    FS PHAR     IVLTFE   SLD I        L I QQ  IQ
aplPKA-1  FS PHSR     IVLV E   HLDIM        L I SY
aplPKA-2  FS SHSR     IILSFE   YLD V        L I PQ  C
aplSAK    LPEYWCTFYAAQVTMALQYLHNCSLLYRDLKPENILLDHLGYLKVTDFGF
bovPKG    FEDSTTR  T C VE FA   SKGII        LI   R  A LV
bovPKC    FK PQAV    EISIG FF  KRGII     LD VM  SE HI IA   M
rabNPKC   FD PRSR    E  S  MF  QHGVI     LD     AE HC LA   M

          151                                                200
bovPKA    AK  K ...R        E     I  SKG NKAVDWWA  V    MA
aplPKA-1  AK  K ...R        E     I  SKG NKAVDWWA  V  T MA
aplPKA-2  AK  K ...R        E     I  SKG NKAVDWWA  V  T MA
aplSAK    DRRVQG...HTWTLCGTPQYLAPEMILNRSYASQWTTGGLGILIYELNAG
bovPKG    AKKI. FGKK   F    E V   I   KGHDISADYWS    M   LT
bovPKC    CKEHMMDGVT R F    D I   I AYQP GKSVDWWAY V L  ML
rabNPKC   CKEGILNGVT T F    D I   ILQELE GPSVDWWA  V M  MM

          201                                                250
bovPKA    YP  FADQ IQI  K  SGKVR..   H SSD K   R LL   L K
aplPKA-1  YP  FADQ IQI  K  SGKVR..   H SSD K   R LL   L K
aplPKA-2  YP  FADQ IQI  K  SGKVR..   H SSD K   R LL   L K
aplSAK    FVPFDHKVPLKLYELIVECRLT..FPSFFKPTLRDLLTNIIQVDVTRRFG
bovPKG    SP  SGPD M T NI LRGIDMIE  KKIAKNAAN IKKLCRDNPSE L
bovPKC    QP   GEDEDE FQS M HNVS..Y KSLSKEAVSICKGLMTKHPGK L
rabNPKC   QP  EADNEDD F S LHDDVL..Y VWLSKEAVSI KAFMTKNPHK L

          251              270
bovPKA     .. KD VN  K  K  AT
aplPKA-1   .. K  VN  K  K  ST
aplPKA-2   .. K  VN  K  K  ST
aplSAK    N..LRNGALDIINHPWFKDT
bovPKG     .. K  VK  QK K  EGF
bovPKC    C..GPE ER VRE AF RRI
rabNPKC   CVAAQ  EDA KQ  F  EI
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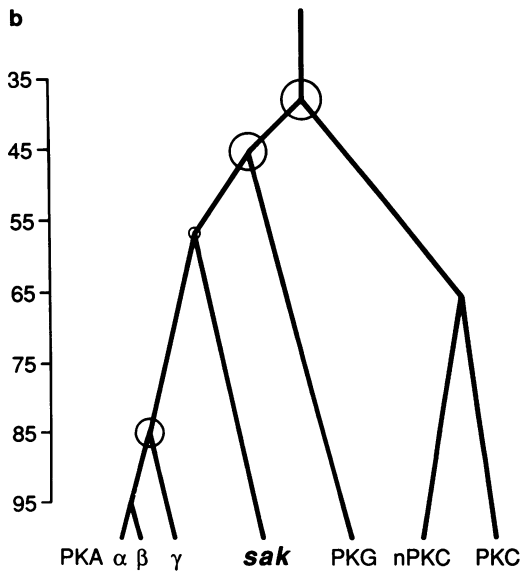

FIG. 3. Comparison of C_{APL-B} with closely related kinases. (a) Amino acid sequence alignment of kinase domains. Kinase domains of C_{APL-B} and various related molecules are aligned (bovPKA, bovine PKA C_α [26]; aplPKA-1, *Aplysia* PKA C_{APL-A1} [3]; aplPKA-2, *Aplysia* PKA C_{APL-A2} [3]; aplSAK, *Aplysia* C_{APL-B} [this work]; bovPKG, bovine PKG [27]; bovPKC, bovine PKCα [24]; rabNPKC, rabbit nPKCε [1, 20, 21]). Residues identical to those in C_{APL-B}, the sequence of which is shown in full, have been omitted. Gaps in the alignments are shown as dots. (b) Phylogenetic tree deduced from the alignment. Additional sequences are human PKA C_β and human PKA C_γ (2). The tree is based on the percent sequence identity obtained from aligned sequences by using the program DISTANCES (GCG package; University of Wisconsin Biotechnology Center). The diameter of the circle at each branch point represents the range of values for all possible comparisons of members of the left branch with members of the right branch.

The presence of the C_{APL-B} polypeptide in mature sperm cells and its rather distant relationship to the C_{APL-A} gene products (Fig. 3b) suggest that it has a role in fertilization, although the possibility of a role in spermatogenesis cannot yet be dismissed. Mature sperm cells have many special properties, including motility with demonstrated chemotaxis in some species and the ability to undergo capacitation and the acrosome reaction. These properties are controlled in part by signals arising in the female reproductive tract (the hermaphroditic organs in the case of *Aplysia* spp). Kinases have been implicated in several of these processes (9). For example, the mouse zona pellucida glycoprotein ZP-3 (which is involved in adhesion and in the induction of the acrosome reaction), sea urchin FSG (which induces the acrosome reaction), and sea urchin egg peptides (which are responsible for motility changes and chemotaxis) all elevate cAMP in cognate spermatozoa. The egg peptides also elevate cGMP concentrations. It is even conceivable that sperm cell kinases are involved in postfertilization events. For example, during sperm nucleus decondensation within the egg, phosphorylation of sperm-derived histones has been demonstrated (10), and a kinase originating from the spermatozoon could be responsible.

The extent of sequence identity of C_{APL-B} with PKA (55 to 57% in the kinase domain) is so low that we cannot, in the absence of experimental data, conclude how the kinase is regulated or infer the nature of its substrates. Because C_{APL-B} is a member of the AGC family and may interact with common cellular substrates, activators, and inhibitors, it is possible that some cellular functions previously associated with PKA, PKG, or PKC actually involve C_{APL-B}.

Nucleotide sequence accession number. The nucleotide sequence of C_{APL-B} cDNA has been submitted to GenBank under accession no. M38049.

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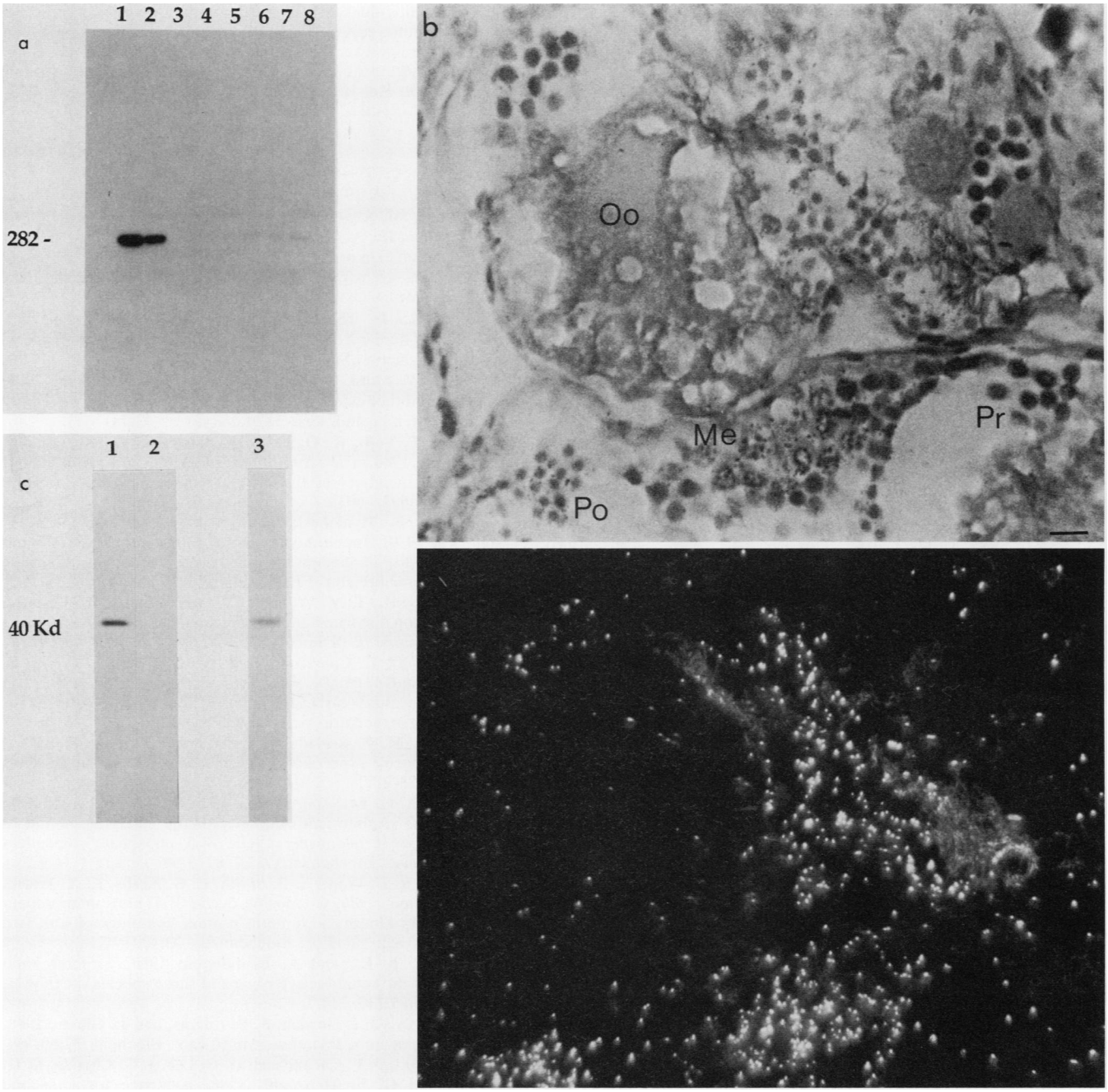

FIG. 4. Localization of C_{APL-B} transcripts and polypeptides in the reproductive system of *A. californica* (clockwise from left). (a) High levels of transcripts are present only in the reproductive system of adult *A. californica*. RNAs from the following sources were amplified by RT-PCR: adult ovotestis poly(A)$^+$ RNA (lane 1), poly(A)$^+$ RNA from a homogenized young adult animal (lane 2), poly(A)$^+$ RNA from a young adult animal from which the entire reproductive tract was surgically removed (lane 3 [all tissue from the ovotestis to the point of attachment of the large hermaphroditic duct to the body wall was excised]), and total RNA extracted from immature animals collected at the same time (lane 4, 2.5 g; lane 5, 2.6 g; lane 6, 2.9 g; lane 7, 3.1 g; and lane 8, 3.3 g). RT-PCR was performed as described in the legend to Fig. 1a. The position of the 282-bp amplification product is shown. (b) In situ hybridization of C_{APL-B} cRNA to ovotestis tissue. Several cell types can be seen in the bright field (top), including an oocyte (Oo) and premeiotic (Pr), meiotic (Me), and postmeiotic (Po) spermatogenic cells. Silver grains, which are more readily visible in the corresponding dark field (bottom), are concentrated over the meiotic and postmeiotic cells. Bar, 10 μm. The probe was ^{35}S-labeled antisense cRNA transcribed from linearized plasmid DNA containing the 463-bp 5′ *Not*I-*Sal*I fragment of cDNA SB-OT-18. After hybridization, preliminary washing, and RNase digestion, the slides were subjected to a 50% formamide wash at 65°C in 0.5 M NaCl with 20 mM 2-mercaptoethanol (2ME for 15 min and then washed in 0.5× SSC–20 mM 2ME for 30 min at 37°C and in 0.1× SSC–20 mM 2ME for 15 min at 37°C before being coated with emulsion. (c) Detection of the C_{APL-B} polypeptide in a homogenate of mature *Aplysia* spermatozoa by immunoblotting. The specificity of OK-C1, an antipeptide antibody against a carboxyl-terminal peptide of C_{APL-B} ([C]EESISISKHDKYPDE), was tested against the C_{APL-B} polypeptide heterologously expressed in Sf9 cells (S. Beushausen, S. Cheley, and H. Bayley, unpublished data). Lane 1, Sf9 cells, OK-C1, and no blocking peptide; lane 2, Sf9 cells and OK-C1, with blocking peptide; lane 3, *Aplysia* sperm homogenate, OK-C1, and no blocking peptide. In additional control experiments, OK-C1 did not react with C_{APL-A1} or C_{APL-A2}

expressed in insect cells (not shown). Sperm was extruded from the small hermaphroditic duct dissected from a mature animal. The cells, which comprise almost entirely mature spermatozoa (28), were washed in phosphate-buffered saline, solubilized in Laemmli loading buffer for 5 min at 95°C, and subjected to electrophoresis in an 11% sodium dodecyl sulfate-polyacrylamide gel (~25 μg of protein per lane). Molecular mass markers were as follows (in daltons): phosphorylase *b* (97,400), bovine serum albumin (66,200), ovalbumin (42,700), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and egg white lysozyme (14,400). Kd, Kilodaltons.

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