BIOLOGY OF REPRODUCTION 43, 46–54 (1990)

Subunits of Cyclic Adenosine 3′, 5′-Monophosphate-Dependent Protein Kinase Show Differential and Distinct Expression Patterns during Germ Cell Differentiation: Alternative Polyadenylation in Germ Cells Gives Rise to Unique Smaller-Sized mRNA Species[1]

OLE ØYEN,[2,3,4] FRODE MYKLEBUST,[3] JOHN D. SCOTT,[5] GARY G. CADD,[6] G. STANLEY McKNIGHT,[6] VIDAR HANSSON,[4] and TORE JAHNSEN[3,4]

Institute of Pathology[3] Rikshospitalet, Oslo 1, Norway, Institute of Medical Biochemistry[4] University of Oslo, Blindern, Oslo 3, Norway
Department of Physiology and Biophysics, and Department of Biological Chemistry[5] University of California Irvine, Irvine, California 92717
Department of Pharmacology[6] University of Washington, Seattle, Washington 98195

ABSTRACT

Cyclic AMP (cAMP) and cAMP-dependent protein kinases (PKAs) are believed to be involved in the regulation of essential spermatozoal functions, such as motility, epididymal maturation, capacitation, and the acrosome reaction. In this study, we document the presence of significant mRNA levels for 5 different PKA subunits (RI_α, RI_β, RII_α, RII_β, and C_α) in germ cells and demonstrate differential expression patterns for these subunits during spermatogenesis. Messenger RNAs for RI (RI_α and RI_β) and C_α appear to be induced at premeiotic germ cell stages, whereas mRNAs for RII (RII_α and RII_β) are first expressed at haploid stages. The individual PKA subunits may convey specific functions in developing germ cells and mature sperm.

The present study, furthermore, demonstrates the presence of unique smaller-sized mRNAs in germ cells compared with somatic cells. Specific, truncated forms of RI_α, RII_α, RII_β, and C_α mRNAs appear to be selected in the germ cells. Our data suggest this to be due to the use of alternative polyadenylation site signals. The selection of shorter mRNA species, with higher stability, may be essential for the delayed translation observed in spermatids. This may ensure certain levels of mRNA for translation at late spermatid stages, after cessation of transcription.

INTRODUCTION

It is well established that cyclic AMP (cAMP) is involved in the regulation of highly specialized spermatozoal functions such as motility, epididymal maturation, capacitation, and the acrosome reaction (Garbers and Kopf, 1980; Tash and Means, 1983). Numerous investigations to elucidate these diverse and complex functions in sperm have focused on the role played by cAMP and its effector enzyme, cAMP-dependent protein kinase (PKA). Also at earlier stages of germ cell differentiation (spermatocytes, spermatids), the presence of significant levels of PKA has been firmly documented (Conti et al., 1983). However, the role of cAMP-dependent regulatory mechanisms during earlier stages of spermatogenesis has remained obscure, although substantial evidence exists suggesting a general role for PKA as a positive effector of growth and differentiation (Russell, 1978).

Recent studies on PKAs, primarily involving cDNA cloning and sequencing, have revealed an unexpected multiplicity in isoforms representing different gene products. Four different regulatory subunits (R) and two different catalytic subunits (C) for PKA have now been identified, at least at the gene/mRNA level. These have been designated RI_α (Lee et al., 1983), RI_β (Clegg et al., 1988), RII_α (Scott et al., 1987), RII_β (Jahnsen et al., 1986; Levy et al., 1988), C_α (Uhler et al., 1986a), and C_β (Showers and Maurer, 1986; Uhler et al., 1986b). RI subunits give rise to type I PKA, whereas RII subunits give rise to type II. In addition, we have recently isolated cDNA for a third catalytic subunit, designated C_γ, from human testis, and demonstrated a testis-specific expression for this subunit (Beebe et al., 1990).

In previous investigations regarding PKAs in germ cells (and PKAs in general), the enzyme complex has more or less been considered one homogenous pool of molecules, and the only distinction possible has been between type I and type II. A selective decrease in type I PKA has been demonstrated with the progress of spermatid development, and type II was found to be the major form present in elongating spermatids (Conti et al., 1983). The newly developed cDNA probes for the different subunits of PKA have enabled us to investigate the expression of each subunit separately at the mRNA level. We have previously reported the cell-specific expression of mRNAs for RI_α (previously de-

Accepted February 16, 1990.
Received August 28, 1989.
[1]This work was supported by the Norwegian Cancer Society (T.J., V.H.), Norwegian Research Council for Science and the Humanities (T.J., V.H.), Torsteds grant (T.J., V.H.), Anders Jahres Foundation for the Promotion of Science (T.J., V.H.), Nordic Insulin Foundation (T.J., V.H.) and grants from the NIH (G.S.M.).
[2]Reprint requests: Ole Øyen, Institute of Pathology, Rikshospitalet, 0027 Oslo 1, Norway.

noted RI), RII_β (previously denoted RII_{51}), and C_α in different testicular cell types (somatic and germinal) (Øyen et al., 1987). Recently, we have demonstrated the high-level expression of a unique mRNA species for RII_α at late stages of spermatogenesis—e.g. during spermatid elongation (Øyen et al., 1988b).

We have now been able to investigate the expression of all known subunits of PKA at the mRNA level by means of highly specific cDNA (and oligonucleotide) probes. In the present study, we report characteristic patterns of expression during spermatogenesis for several subunits, and we demonstrate the presence of a unique alternative polyadenylation mechanism in germ cells that favors smaller mRNA species.

MATERIALS AND METHODS

Preparation of various tissues and isolated testicular cells. Tissue samples were taken from rat liver, heart, lung, ovary (hypophysectomized and estradiol/FSH-treated rats [Jahnsen et al., 1986]), and brain. Human testicular tissue (nonpathologic) was obtained at surgery from a 14-yr-old patient. All tissue samples were frozen at once in liquid N_2 and stored at −75°C for later RNA extraction.

Cultured Sertoli cells (control and stimulated with 0.1 mM dibutyryl-cAMP), cultured peritubular cells, Leydig cell tumor tissue, and whole testis tissue from rats of different ages were prepared as previously described by Øyen et al. (1987).

Preparation of rat germinal cell fractions. Germ cells were isolated from seminiferous tubules of 32- and 44-day-old rats. A cell preparation was obtained by consecutive collagenase, trypsin, DNAse, and mechanical treatment (Øyen et al., 1987). Pachytene spermatocytes (PS), round spermatids (RST), and elongating spermatids (ES; present only in 44-day-old rats) were then isolated by the StaPut unit-gravity sedimentation method using a BSA gradient, essentially as described by Grootegoed et al. (1977). The germ cells were examined both by phase-contrast microscopy and by regular light microscopy after fixation and staining. The purities of these germ cell fractions were evaluated by counting cells: PS, 32 days, 90–95%; RST, 32 days, 85–90%; PS, 44 days, 75–80%; RST, 44 days, 65–70% (contamination mostly ES); ES, 45–55% (contamination mostly RST).

Preparation of total RNA. RNA extraction from whole testes and from isolated testicular cells was performed as previously described (Øyen et al., 1987) by homogenization in guanidinium isothiocyanate. Total RNA was isolated by centrifugation through a cesium chloride gradient and purified by phenol/chloroform extractions.

Complementary DNA probes. The mouse RI_α probe was a 0.6 kb *Pst*I cDNA fragment (McKnight GS; unpublished data) from inside the open reading frame (ORF), and the mouse RI_β probe was a 1.5 kb *Eco*RI fragment (Clegg et al., 1988) containing the entire ORF. The rat RII_α cDNA was a 0.7 kb *SAlI-Bgl*II fragment (Scott et al., 1987) from inside the ORF, whereas the probe used for RII_β was a 1.5 kb rat cDNA containing both 3′ coding (1.2 kb) and noncoding (0.3 kb) regions (Jahnsen et al., 1986). Furthermore, we used a 0.6 kb *Eco*RI fragment of mouse C_α containing 3′ coding region and about 160 bp of 3′ untranslated sequence (Uhler et al., 1986a). The bovine C_β probe was a 1.5 kb *Dra*I fragment, representing 1 kb of coding sequence and 0.5 kb 3′ noncoding sequence (Showers and Maurer, 1986).

Northern analysis. The samples were electrophoresed on a 1.5% agarose gel containing formaldehyde as denaturing agent and with recirculating 20 mM sodium-phosphate running buffer (Øyen et al., 1987). Twenty micrograms total RNA was used in each lane, separated on the gel, and transferred to a nylon filter (Biotrans; ICN Biomedicals, Costa Mesa, CA) by capillary blotting technique. The resulting filter was prehybridized in 50% formamide, 5X SSC (Maniatis et al., 1982), 5X Denhardt's solution, 50 mM sodium phosphate (pH 6.5), 0.1% sodium dodecyl sulfate (SDS), and 250 μg/ml denatured salmon sperm DNA, at 42°C, and hybridized under the same conditions using nick-translated (Øyen et al., 1987) cDNA probes for the different subunits of PKA. Washing was performed with 0.1X–0.5X SSC at 50°C, and the filter was finally autoradiographed using Hyperfilm MP (Amersham, UK). Messenger RNA sizes were estimated by comparison to RNA standards (Bethesda Research Laboratories, Bethesda, MD).

Hybridization with C_α-specific oligonucleotide probe. An oligo (40 mer) corresponding to the terminal 3′ nontranslated region of human C_α cDNA (Maldonado and Hanks, 1988), nts 2462–2501, was purchased from Genetic Designs Inc. (Houston, TX). The terminal 3′ region of C_α is very well conserved among species (Uhler et al., 1986a). End-labeling was performed as described by Maxam and Gilbert (1977) using [γ-^{32}P]-ATP (Amersham, PB10218) and T4 polynucleotide kinase. Northern nylon filters were probed under the following prehybridization/hybridization conditions: 40% formamide, 5X SCC, 5X Denhardt's solution, 50 mM sodium phosphate (pH 6.5), 0.1% SDS, 250 μg/ml denatured salmon sperm DNA, 50 μg/ml tRNA, at 42°C. Filters were then washed with 0.5X–1X SSC at 50°C.

RESULTS

Cell-specific mRNA Expression in Rat Testis

Figure 1 shows the mRNA expression pattern of the various PKA subunits in different rat testicular cells and in whole testis. RI_β has been found to be expressed in a highly tissue-specific manner, and has been denoted a brain-specific form of PKA regulatory subunits (Clegg et al., 1988). This Northern blot demonstrated the single 2.6 kb RI_β mRNA also to be present at significant levels in germ cells (PS, Lane 3; RS, Lane 4), giving rise to a corresponding signal in whole testis of postpubertal rats (Lane 9). The RI_β cDNA probe

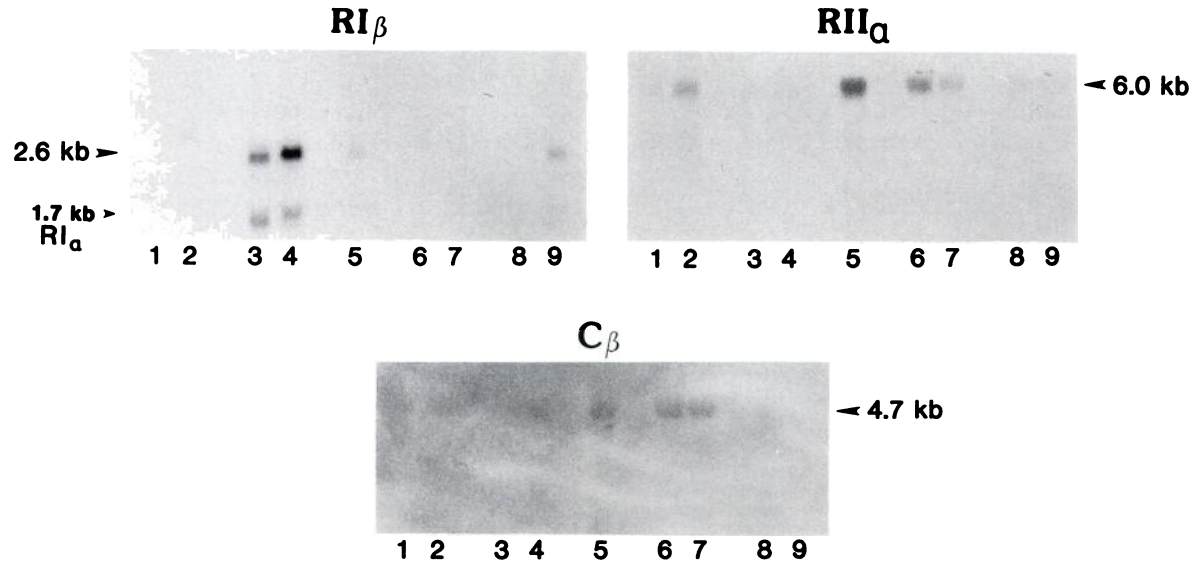

FIG. 1. Northern blot showing levels of mRNA for RI_β, RII_α, and C_β in different testicular cell types. Total RNA was extracted from various cells/tissues representing the different cell types in rat testis. *Lanes 1* and *2:* Cultured Sertoli cells; control (*Lane 1*) and stimulated with 0.1 mM dibutyryl-cAMP for 48 h (*Lane 2*). *Lanes 3* and *4:* StaPut germinal cell fractions: pachytene spermatocytes (PS, *Lane 3*) and round spermatids (RST, *Lane 4*). *Lane 5:* Cultured peritubular cells. *Lanes 6* and *7* (similar): Rat Leydig cell tumor tissue. *Lanes 8* and *9:* Whole rat testis: 20 days (*Lane 8*) and 30 days (*Lane 9*) of age. Twenty micrograms of total RNA was loaded in each lane and the resulting filter was probed succesively with ^{32}P-labeled cDNAs for RI_β, RII_α, and C_β and autoradiographed.

also detected a lower band representing cross-hybridization with the RI_α 1.7 kb mRNA (Øyen et al., 1987) (Figs. 2 and 3), which is also induced in germ cells. In addition, a very faint but distinct RI_β mRNA band was detected in the peritubular cells (Lane 5). These cells have been cultured for 13 days, which excludes the possibility that the RI_β signal was due to germ cell contamination.

The RII_α 6.0 kb mRNA, which is expressed in most tissues (Scott et al., 1987; Øyen et al., 1987), was detected in all testicular cells, but in variable amounts (Fig. 1). The strongest RII_α single was seen in peritubular cells. Intermediate levels were detected in stimulated Sertoli cells (0.1 mM dibutyryl-cAMP; Lane 2) and Leydig cell tumor tissue (LCT; Lanes 6 and 7), whereas very faint bands were observed in germ cells (may represent contamination of somatic cells in the germ cell fractions). We have previously reported the differential regulation of PKA subunits by cAMP analogues in Sertoli cells (Øyen et al., 1988a).

For C_β, low levels of the 4.7 kb mRNA were detected in peritubular cells and LCT. In germ cells and Sertoli cells, C_β was found to be below the level of detection (under our labeling/hybridization conditions). We have previously shown the mRNA expression patterns for RI_α, RII_β, and C_α in rat testicular cells (Øyen et al., 1987).

Developmental changes in mRNA expression in whole testis. At early puberty (15–25 days of age in rats) there is an exponential increase in germ cells, which at later ages dominate the testis and dilute the signals from somatic cells in whole testis preparations. In this way, the cellular localization (somatic cells vs. germ cells) of mRNAs in the testis can be predicted from the age variation pattern. According to this (Fig. 2), expression of the two larger RI_α mRNAs (2.9 kb and 3.2 kb), which have been found to be the most abundant forms in somatic cells (Øyen et al., 1987), decreased with age, whereas there was a marked increase in expression of the usually fainter 1.7 kb RI_α mRNA from 25–30 days of age, indicating an induction of this mRNA species in meiotic germ cells. The specific germ cell localization of the RI_β 2.6 kb mRNA was supported by developmental studies, which demonstrated the appearance of this mRNA from 30 to 35 days of age.

Accordingly, the 6.0 kb RII_α mRNA and the 3.2 kb RII_β mRNA, both found as dominant forms in somatic cells, showed declining levels with age. The 2.4 kb C_α mRNA, in contrast, demonstrated an enrichment from 30 to 35 days of age, and a unique RII_α mRNA species (2.2 kb) appeared at high levels from 40 days of age (Øyen et al., 1988b). In addition, unique smaller-sized mRNAs for RII_β and C_α appeared in postpubertal testis. A 1.6 kb RII_β signal (below the faint 1.9 kb mRNA previously described (Jahnsen et al., 1986) was detected at variable intensities beyond the age of 35 days. A C_α mRNA band, located closely below the usual 2.4 kb mRNA, appeared from 30 to 35 days of age (we will refer to this unique band as the 2.3 kb mRNA). The C_β 4.7 kb mRNA was detected very faintly in whole testis preparations and showed a declining tendency with age (not shown).

Messenger RNA expression in isolated germ cell fractions. The Northern blots on the germ cell fractions (Fig. 3) should be interpreted in light of the estimated purities of these fractions (listed in *Materials and Methods*). The 1.7 kb RI_α mRNA, as suggested from the developmental studies,

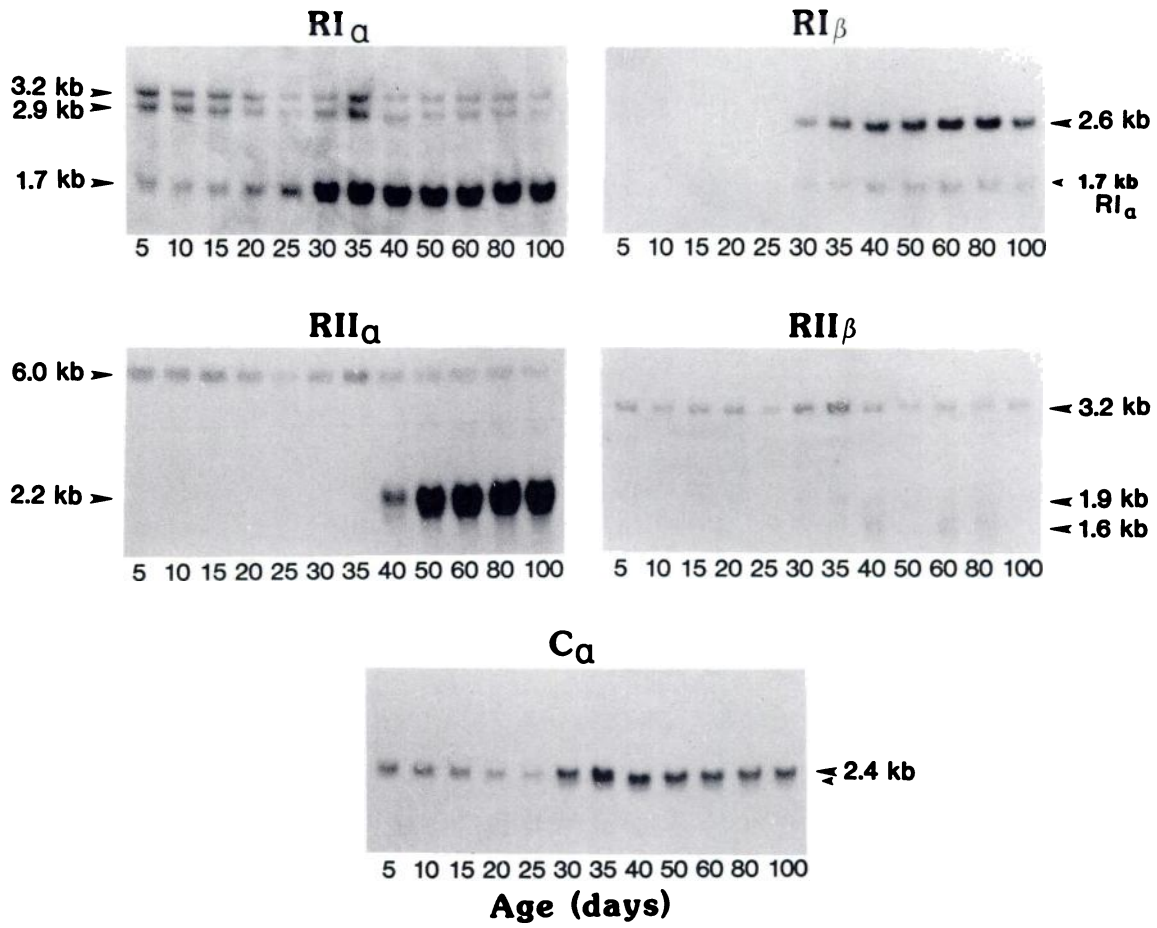

FIG. 2. Northern blot showing levels of mRNA for RI_α, RI_β, RII_α, RII_β, and C_α in testis from rats of different ages. RNA was extracted from whole rat testis of animals of various ages. Twenty micrograms total RNA was loaded in each lane on the gel and the resulting filter was probed succesively with nick-translated cDNAs for RI_α, RI_β, RII_α, RII_β, and C_α. The *abscissa* shows increasing rat ages. Approximate mRNA sizes are indicated.

was found to be present at high levels in PS (meiotic cells) and RST (postmeiotic cells) isolated from both 32- and 44-day-old rats, while declining levels were found at the ES stage (ES present only in 44-day-old rats). For the brain- and germ cell-specific RI_β subunit, a similar pattern was demonstrated; the 2.6 kb mRNA was found at significant levels in both PS and RST, and declined at the ES stage. The very abundant 2.2 kb RII_α mRNA was located primarily in the RST and ES fractions of 44-day-old rats. The presence of this band in the PS fraction of 44-day-old rats could be accounted for by ES contamination of this fraction. The 2.2 kb RII_α signal was not present in the more purified RST fraction from 32-day-old rats, and the developmental studies (Fig. 2) suggested the small-sized RII_α mRNA to be induced at the ES (or late RST) stage. In addition, the RII_α cDNA detected a faint 3.1 kb signal (Øyen et al., 1988b), which may represent a nuclear RNA precursor.

The presence of a 1.6 kb RII_β mRNA in germ cells, as suggested from the developmental studies, was confirmed. This smaller RII_β mRNA was found predominantly in the RST fraction. The presence of a smaller 2.3 kb C_α-related mRNA was also clearly demonstrated in the germ cells. This mRNA showed the same expression pattern as the usual 2.4 kb C_α mRNA, but was less abundant. Both C_α mRNAs exhibited a pattern of expression similar to that found for the RI_α 1.7 kb mRNA: significant expression both in PS and RST and a marked decline in ES. C_β mRNA was below the level of detection in the isolated germ cells.

C_α-Related mRNAs. A C_α-specific oligonucleotide, corresponding to the far 3′ region of the human C_α cDNA (Maldonado and Hanks, 1988), detected (Fig. 4) both the usual "somatic" 2.4 kb mRNA and the postpubertal 2.3 kb band (as well as the corresponding human C_α 2.8 kb mRNA). In addition, a 1.8 kb mRNA was detected faintly by the C_α cDNA probe in some blots (Fig. 5), but was only seen in testis, as opposed to the rather ubiquitous 2.4 kb mRNA. A human testis-specific mRNA of the same size (1.8 kb) has been shown to represent a third C subunit (C_γ; Beebe et al., 1990).

DISCUSSION

This study documents the presence of significant mRNA levels for 5 different PKA subunits in germ cells, and dem-

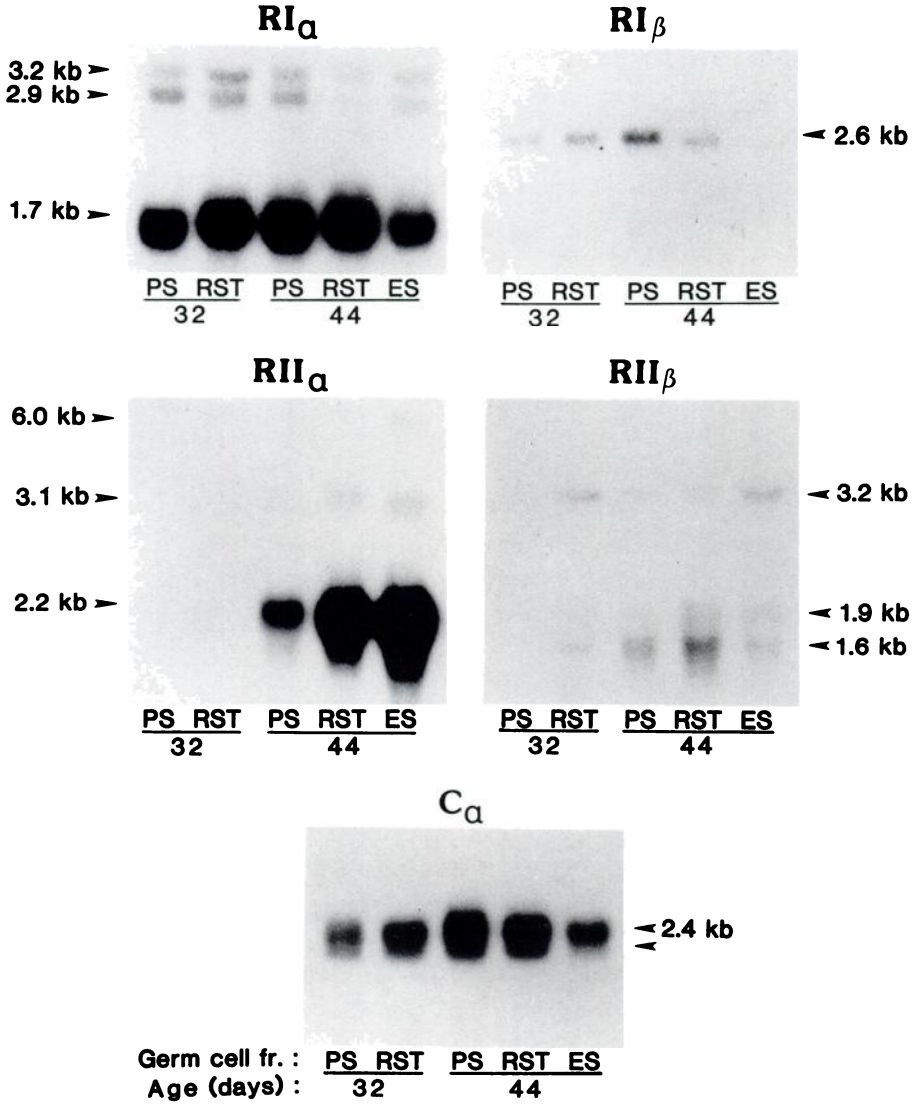

FIG. 3. Northern blot showing mRNA levels for subunits of PKA in different germ cell fractions (*PS:* pachytene spermatocytes; *RST:* round spermatids; *ES:* elongating spermatids). RNA was extracted from germinal cell fractions obtained by the StaPut unit-gravity sedimentation method on testes of 32-day-old rats and 44-day-old rats (ES present only in 44-day-old rats). Twenty micrograms total RNA was loaded in each lane on the gel and the resulting filter was probed with nick-translated cDNAs for RI_α, RI_β, RII_α, RII_β, and C_α.

onstrates differential expression patterns for these subunits during spermatogenesis. Some of these subunits (RI_β and RII_β) have been found to be expressed in a very limited number of cell types.

When taking into account both the developmental studies and the studies performed on isolated germ cell fractions, rather conclusive statements can be made regarding the stage-specific expression of the different PKA subunits during germ cell differentiation. The RI_α 1.7 kb, RI_β 2.6 kb, and C_α 2.4/2.3 kb mRNAs were induced at premeiotic/meiotic stages, as they appeared/increased from Days 25 to 30 in the developmental studies and clearly were present in PS germ cell fractions. Their expression tended to increase further in RST, whereas a marked decline was found at the ES stage. In fact, most or all of the RI_α 1.7 kb and C_α signals seen in the ES fraction can be accounted for by RST contamination of this fraction. The high-level RII_α 2.2 kb mRNA was found to be induced postmeiotically, at the ES (or late RST) stage, since it first appeared at 40 days of age. The data on the less abundant 1.6 kb RII_β mRNA also indicated postmeiotic induction, as it first appeared at 35 days of age and was undetectable in the PS fraction of 32-day-old rats. However, this RII_β mRNA seemed to be expressed predominantly in RST and its expression declined in ES. C_β mRNA did not seem to be expressed at detectable levels in germ cells.

Overall, it appears that the mRNAs for the RIs and C_α are induced at premeiotic germ cell stages, whereas mRNAs for the RIIs are first expressed at haploid stages. Furthermore, the major part of the RII_α 2.2 kb mRNA expression takes place at later spermatid stages compared with the RII_β 1.6 kb mRNA. These findings are in agreement with previous

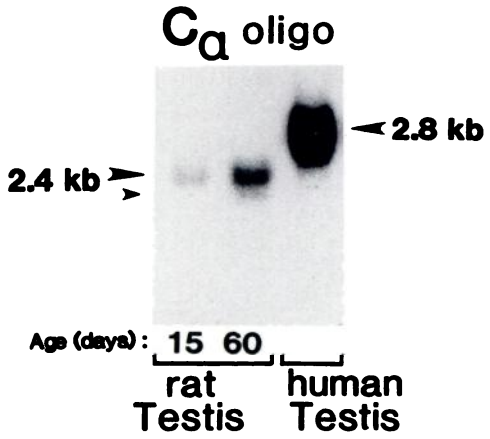

FIG. 4. Northern blot showing mRNAs detected by a C_α-specific oligonucleotide probe. Total RNA was extracted from rat (15 and 60 days old) and human testes (14-year-old patient) and subjected to Northern analysis. The resulting filter was hybridized with the end-labeled C_α-specific oligonucleotide (40 mer; see *Materials and Methods*). The smaller *arrow* on the *left* indicates the fainter 2.3 kb band seen in postpubertal rats.

studies on kinase activity demonstrating that type I PKA is the dominating form in PS and RST, whereas type II PKA is the major form in ES (Conti et al., 1983).

What are the functional implications of these characteristic and differential expression patterns for PKA subunits during spermatogenesis? The recently discovered diversity in isoforms of PKA offers a new model for cAMP signal transduction. Various isoforms may differentiate cAMP responses at the level of the kinase. Different regulatory subunits may localize the kinase activity to specific subcellular compartments/structures (Vallee et al., 1981; Lohmann et al., 1984; De Camilli et al., 1986) where certain substrates are targets for phosphorylation; this could be related to the heterogeneity observed in the aminoterminal part of the regulatory subunits (Levy et al., 1988; Øyen et al., 1989), whereas the various catalytic subunits may differ in their substrate specificities. This view may help explain the great flexibility of the cAMP signalling system and the diverse effects observed in cells, as in the spermatozoa, when stimulated by cAMP. So far, conclusive evidence demonstrating separate functions attached to a specific PKA subunit, has not been provided. However, specific interactions between type II PKA and various subcellular structures have been documented (Vallee et al., 1981; Lohmann et al., 1984). Concerning germ cells particularly, a characteristic subcellular localization has been demonstrated for RI and RII in sperm by immunogold electron microscopy (Pariset et al., 1989) and by cell fractionation studies (Horowitz et al., 1984; Atherton et al., 1985). The interaction of RII with the sperm flagellum has been well documented (Horowitz et al., 1984, 1988) and the molecular mass of this flagellar RII (56–57 kDa) (Horowitz et al., 1988; Paupard et al., 1988) is compatible with RII_α. It seems very likely, then, that the unique, abundant RII_α 2.2 kb mRNA induced at late spermatogenic stages encodes the RII form interacting with flagellar structures. Thus, RII_α may be responsible for anchoring kinase activity to the sperm flagella, where cAMP-dependent phosphorylation of certain substrates is believed to be essential for sperm motility (Tash et al., 1986; Brokaw, 1987).

RI has been shown to be predominant in the membrane fraction of spermatozoa (Horowitz et al., 1984), and immunogold electron microscopy has suggested that RI is particularly confined to the acrosomal (head) membranes (Pariset et al., 1989). However, the data on RI are still too scarce to suggest any specific functions in sperm or to differentiate RI_α/RI_β at the protein and functional levels.

As concerns the catalytic subunits, only C_α seems to be expressed in significant amounts in rat germ cells. However, cDNAs for a third form of C, designated C_γ, have recently been isolated from human testis (Beebe et al., 1990), and detectable levels of the 1.8 kb C_γ mRNA was found only

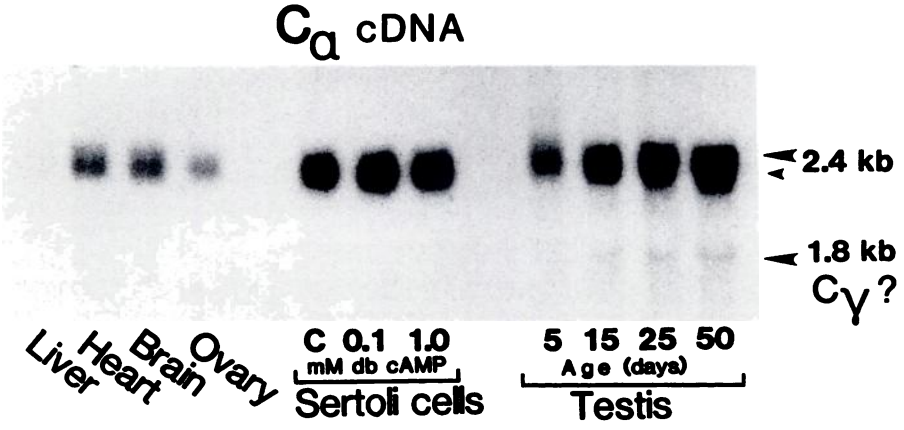

FIG. 5. Northern blot showing C_α-related mRNAs in various rat cells and tissues using C_α cDNA as probe. Total RNA was extracted from the following rat cells and tissues: liver, heart, brain, lung, and ovary (hypophysectomized and estradiol/FSH-treated rats), Sertoli cells (control [*C*] and stimulated with 0.1 or 1.0 mM dibutyryl-cAMP for 48 h) and testis from rats of various ages. Twenty micrograms of total RNA was loaded in each lane and the resulting filter was probed with the nick-translated C_α cDNA (mouse). A possible (cross-hybridizing) C_γ mRNA (1.8 kb) seen in rat testis is indicated.

in human testicular tissue. C_γ showed cross-hybridization with C_α in the human system. A C_α-related mRNA of the same size as the low-abundant human C_γ mRNA (1.8 kb) was demonstrated in rat testis (Fig. 5) and may represent rat C_γ. Future studies will reveal if this recently discovered subunit is also expressed in the germ cells.

Apart from the proposed functions for PKA isoforms in mature sperm, PKA may play a role as a positive effector of growth and differentiation (Russell, 1978) at earlier stages of spermatogenesis. However, no evidence has yet been provided concerning PKA function in early germ cells.

This study documents the induction of unique smaller-sized mRNAs in germ cells as compared with somatic cells. The 2.2 kb RII_α, 1.6 kb RII_β, and 2.3 kb C_α mRNAs appear to be expressed exclusively in the germ cells. The 1.7 kb RI_α mRNA, shown to be highly enriched in germ cells, has also been detected in various somatic cells; however, in somatic cells, the two larger RI_α mRNAs (2.9 kb and 3.2 kb) have been the dominant ones (Øyen et al., 1987). From these observations, we postulate the presence of alternative polyadenylation mechanisms in germ cells, favoring smaller mRNA species. The data available so far disprove the possibility that these unique mRNAs encode alternate proteins by representing different genes or by alternate splicing. Hybridization data with 3′ fragments (McKnight GS; unpublished data) indicate that the three RI_α mRNAs are transcripts of a single gene and represent different polyadenylation sites. A human testis RII_α cDNA (Øyen et al., 1989) corresponding to the unique rat 2.2 kb RII_α mRNA has been shown to contain the same coding region as the larger somatic transcript. Furthermore, the smaller 2.3 kb C_α mRNA was also detected (Fig. 4) by an oligonucleotide from the 3′ region of the C_α cDNA.

Recently, a germ cell-specific *c-abl* mRNA (also smaller than the usual somatic transcripts) was shown not to contain the usual polyadenylation site signal (AAUAAA) (Meijer et al., 1987). However, a resembling hexanucleotide (UACAAA) was found 12 nucleotides upstream of the poly(A)-tail (Fig. 6). It should be emphasized at this point that less-conserved sequence elements, G/T-clusters (downstream of the poly(A) addition site), and a CAYUG (Y = pyrimidine residue) motif (upstream or downstream) also are believed to play a role in cleavage/polyadenylation (Birnstiel et al., 1985). Such sequences were found in the germ cell-specific *c-abl* mRNA.

Furthermore, in human testis RII_α cDNA (Øyen et al., 1989), shown to be analogous to the germ cell-specific rat RII_α mRNA (2.2 kb), the usual polyadenylation site signal was also absent (Fig. 6); and 18 nucleotides upstream of the poly(A)-tail, the closely resembling hexanucleotide UAUAAA was found. Neither of these possible polyadenylation site signals in germ cell-specific mRNAs, UACAAA in *c-abl* and UAUAAA in RII_α, has been reported previously (Birnstiel et al., 1985).

An RII_β cDNA (Fig. 6), which corresponds well with the size of the 1.6 kb RII_β mRNA seen in spermatids, has been cloned from rat ovary tissue (Jahnsen et al., 1986). However, a 1.6 kb mRNA has not been found at detectable levels in rat ovary, nor in any other tissue other than testis. This RII_β cDNA did not contain the usual polyadenylation site signal, but the resembling hexanucleotide AUUAAA was present.

A variant polyadenylation site signal (GAUAAA) (Fig. 6) has also been reported in a calmodulin cDNA (prCM79; Sherbany et al., 1987) corresponding to a smaller-sized (0.75 kb) mRNA in rat brain and found to be significantly less abundant than the predominant 2.2 kb mRNA. Interestingly, this shorter transcript was found to be the predominant mRNA species expressed in spermatids (Slaughter and Means, 1989; here called CaM III 1.0 kb).

The induction of unique mRNA species for several different PKA subunits, and the data referring to the presence of atypical polyadenylation site signals, strongly suggests the presence of alternative polyadenylation mechanisms in germ cells. Furthermore, in each of these instances, smaller-sized transcripts are generated. It seems likely that the polyadenylation machinery in germ cells is able to accept alternative polyadenylation site signals, which are preferred when they are located 5′ to the usual (somatic) polyadenylation site signal. Evidence has been presented suggesting that small nuclear ribonucleoprotein particles (RNPs) are involved in cleavage/polyadenylation (Birnstiel et al., 1985), perhaps by partial complementarity between the RNA moiety of the RNPs and sequences surrounding the poly(A) addition site (Berget, 1984). This leads to the speculation that specific snRNPs in germ cells define alternative polyadenylation site signals.

What functional reasons would there be for germ cells to favor alternative mRNA species? Sequences within the 3′ nontranslated region of mRNAs are involved in the regu-

human RII_α 2.0 kb .....TGATACAAAGTCCAAAG<u>TATAAA</u>CATGCTCCTTTCCTCTC(A)$_n$
rat RII_β 1.6 kb .....CATGTTTAAGAAGATA<u>ATTAAA</u>AGATGTACTCATAGGCCG(A)$_n$
mouse c-abl 4.0 kb .....ACTGTACCTGCACCTTTGATGCT<u>TACAAA</u>CTGTCCCCGAG(A)$_n$
rat calmodulin 0.75 kb.....ATTGACTGAGAATCT<u>GATAAA</u>GCAACAAAAGATTTGTCCC(A)$_n$

FIG. 6. Putative polyadenylation site signals in four different mRNAs (cDNAs) found to be uniquely expressed in germ cells. The last 40 nucleotides before the poly(A)-tail, with the putative polyadenylation site signals *underlined*, are shown for human RII_α cDNA (Øyen et al., 1989), rat RII_β cDNA (Jahnsen et al., 1986), mouse *c-abl* cDNA (Meijer et al., 1987), and rat calmodulin (prCM79) cDNA (Sherbany et al., 1987). The corresponding mRNAs (sizes indicated) are uniquely expressed in germ cells and all are smaller than the mRNA species predominating in somatic cells. No hexanucleotide corresponding to the canonical AATAAA is found in these sequences.

lation of transcript stability and half-life (Shapiro et al., 1987; Strickland et al., 1988). Furthermore, sequence motifs (AU-rich) in the 3′ nontranslated region of various transiently expressed genes have been demonstrated to be responsible for destabilization of the transcripts (Shaw and Kamen, 1986). Concerning the *c-abl* transcripts, data indicated a higher stability of the shorter testis-specific *c-abl* mRNA compared with the somatic transcripts.

This would suggest that smaller-sized mRNAs are favored in the germ cells because of higher stability and longer half-life. RNA transcription is not detected in spermatids beyond the stage of nuclear condensation (step 12 of spermiogenesis) (Geremia et al., 1977). Therefore, protein production, mediating the differentiation processes taking place at later stages, must depend on mRNA made at an earlier stage. Several investigators have demonstrated a characteristic translational delay in germ cells (Sinclair and Dixon, 1982; Heidaran and Kistler, 1987). Mouse protamine mRNAs appear at early haploid stages, but remain translationally inactive for several days before the protein is detectable from beyond the stage of nuclear condensation (Hecht, 1986; Kleene et al., 1984). In light of these characteristic germ cell features, the selection of particularly stable mRNA species may be essential to ensure certain levels of mRNA for translation at late spermatid stages—after the cessation of transcription. Thereby, adequate levels of proteins, necessary for late spermatid differentiation and mature sperm functions, may be maintained.

In future experiments we will need tools to study the individual PKA subunits at the protein and functional levels, to explore the possible discrete functions conveyed by each subunit in the germ cells. Efforts are being made to distinguish the various subunits by means of highly specific antibodies, and transgenic animal studies may help us elucidate specific functions. To investigate more closely the alternative polyadenylation in germ cells, the region of the poly(A) addition sites should be characterized in detail in each of the genes. However, the specific mechanisms giving rise to alternate transcripts in germ cells will probably have to await elucidation until more is known about the general mechanisms involved in cleavage/polyadenylation.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Mss. Gro Knutsen, Guri Opsahl, Anne Keiserud, and Inger Ronander.

REFERENCES

Atherton RW, Khatoon S, Schoff PK, Haley BE, 1985. A study of rat epididymal sperm adenosine 3′, 5′-monophosphate-dependent protein kinases: maturation differences and cellular location. Biol Reprod 32:155–71

Beebe JS, Øyen O, Sandberg M, Frøysa A, Hansson V, Jahnsen T, 1990. Molecular cloning of a tissue specific protein kinase (C_γ) from human testis—representing a third isoform for the catalytic subunit of cAMP-dependent protein kinase. Mol Endocrinol (In press)

Berget SM, 1984. Are U4 small nuclear ribonucleoproteins involved in polyadenylation. Nature 309:179–82

Brokaw CJ, 1987. A lithium-sensitive regulator of sperm flagellar oscillation is activated by cAMP-dependent phosphorylation. J Cell Biol 105:1789–98

Clegg CH, Cadd GG, McKnight GS, 1988. Genetic characterization of a brain-specific form of the type I regulatory subunit of cAMP-dependent protein kinase. Proc Natl Acad Sci USA 85:3703–07

Conti M, Adamo S, Geremia R, Monesi V, 1983. Developmental changes of cyclic adenosine monophosphate-dependent protein kinase activity during spermatogenesis in the mouse. Biol Reprod 28:860–69

De Camilli P, Moretti M, Donini SD, Walter U, Lohmann SM, 1986. Heterogenous distribution of the cAMP receptor protein RII in the nervous system: evidence for its intracellular accumulation on microtubules, microtubule-organizing centers, and in the area of the Golgi complex. J Cell Biol 103:189–203

Garbers DL, Kopf GS, 1980. The regulation of spermatozoa by calcium cyclic nucleotides. Adv Cyclic Nucleotide Res 13:251–306

Geremia R, Boitani C, Conti M, Monesi V, 1977. RNA synthesis in spermatocytes and spermatids and preservation of meiotic RNA during spermiogenesis in the mouse. Cell Differ 5:343–55

Grootegoed JA, Grollè-Hey AH, Rommerts FFG, Van der Molen HJ, 1977. Ribonucleic acid synthesis in vitro in primary spermatocytes isolated from rat testis. Biochem J 168:23–31

Hecht NB, 1986. Evidence for haploid-specific genes in the mammalian testis. In: Stefanini M et al. (eds.), Molecular and Cellular Endocrinology of the Testis. Amsterdam: Elsevier Science Publishers B.V. (Biomedical Division), pp. 199–213

Heidaran MA, Kistler WS, 1987. Transcriptional and translational control of the message for transition protein 1, a major chromosomal protein of mammalian spermatids. J Biol Chem 262:13309–15

Horowitz JA, Toeg H, Orr GA, 1984. Characterization and localization of cAMP-dependent protein kinases in rat caudal epididymal sperm. J Biol Chem 259:832–38

Horowitz JA, Wasco W, Leiser M, Orr GA, 1988. Interaction of the regulatory subunit of a type II cAMP-dependent protein kinase with mammalian sperm flagellum. J Biol Chem 263:2098–104

Jahnsen T, Hedin L, Kidd VJ, Beattie WG, Lohmann SM, Walter U, Durica J, Schulz TZ, Schiltz E, Browner M, Goldman D, Ratoosh SL, Richards JS, 1986. Molecular cloning, cDNA structure and regulation of the regulatory subunit (RII_{51}) of type II cAMP-dependent protein kinase from rat granulosa cells. J Biol Chem 261:12352–61

Kleene KC, Distel RJ, Hecht NB, 1984. Translational regulation and deadenylation of a protamine mRNA during spermiogenesis in the mouse. Dev Biol 105:71–79

Lee DC, Carmichael DF, Krebs EG, McKnight GS, 1983. Isolation of a cDNA clone for the type I regulatory subunit of bovine cAMP-dependent protein kinase. Proc Natl Acad Sci USA 80:3605–12

Levy FO, Øyen O, Sandberg M, Taskén K, Eskild W, Hansson V, Jahnsen T, 1988. Molecular cloning, complementary deoxyribonucleic acid structure and predicted full-length amino acid sequence of the hormone-inducible regulatory subunit of 3′:5′-cyclic adenosine monophosphate-dependent protein kinase from human testis. Mol Endocrinol 2:1364–73

Lohmann SM, De Camilli P, Einig I, Walter U, 1984. High-affinity binding of the regulatory subunit (RII) of cAMP-dependent protein kinase to microtubule-associated and other cellular proteins. Proc Natl Acad Sci USA 81:6723–27

Maldonado F, Hanks SK, 1988. A cDNA clone encoding human cAMP-dependent protein kinase catalytic subunit C_α. Nucleic Acids Res 16:8189–90

Maniatis T, Fritsch EF, Sambrook J, 1982. Molecular Cloning (A Laboratory Manual). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory

Maxam AM, Gilbert W, 1977. A new method for sequencing DNA. Proc Natl Acad Sci USA 74:560–64

Meijer D, Hermans A, von Lindern M, van Agthoven T, de Klein A, Mackenbach P, Grootegoed A, Talarico D, Della Valle G, Grosveld G, 1987. Molecular characterization of the testic-specific c-abl mRNA in mouse. EMBO J 6:4041–48

Øyen O, Frøysa A, Sandberg M, Eskild W, Joseph D, Hansson V, Jahnsen T, 1987. Cellular localization and age-dependent changes in mRNA for cAMP-dependent protein kinases in rat testis. Biol Reprod 37:947–56

Øyen O, Myklebust F, Scott JD, Hansson V, Jahnsen T, 1989. Human testis cDNA for the regulatory subunit RII_α of cAMP-dependent protein kinase encodes an alternate aminoterminal region. FEBS Lett 246:57–64

Øyen O, Sandberg M, Eskild W, Levy FO, Knutsen G, Beebe SJ, Hansson V, Jahnsen T, 1988a. Differential regulation of mRNAs for specific subunits of cAMP-dependent protein kinase by cAMP in rat Sertoli cells. Endocrinology 122:2658–66

Øyen O, Scott JD, Cadd GG, McKnight GS, Krebs EG, Hansson V, Jahnsen T, 1988b. A unique mRNA species for a regulatory subunit of cAMP-dependent protein kinase is specifically induced in haploid germ cells. FEBS Lett 229:391–94

Pariset C, Feinberg J, Dacheux J-L, Øyen O, Jahnsen T, Weinman S, 1989. Differential expression and subcellular localization for subunits of cAMP-dependent protein kinase during ram spermatogenesis. J Cell Biol 109:1195–1205

Paupard MC, MacLeod J, Wasco W, Orr GA, 1988. Major 56,000-dalton, soluble phosphoprotein present in bovine sperm is the regulatory subunit of a type II cAMP-dependent protein kinase. J Cell Biochem 37:161–75

Russell DH, 1978. Type I cAMP-dependent protein kinase as a positive effector of growth. Adv Cyclic Nucleotide Res 9:493–506

Scott JD, Glaccum MB, Zoller MJ, Uhler MD, Helfman DM, McKnight GS, Krebs EG, 1987. The molecular cloning of a type II regulatory subunit of the cAMP-dependent protein kinase from rat skeletal muscle and mouse brain. Proc Natl Acad Sci USA 84:5186–92

Shapiro JS, Blume JE, Nielsen DA, 1987. Regulation of messenger RNA stability in eucaryotic cells. Bioessays 6:221–26

Shaw G, Kamen R, 1986. A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell 46:659–67

Sherbany AA, Parent AS, Brosius J, 1987. Rat calmodulin cDNA. DNA 6:267–72

Showers MO, Maurer RA, 1986. A cloned bovine cDNA encodes an alternate form of the catalytic subunit of cAMP-dependent protein kinase. J Biol Chem 261:16288–91

Sinclair GD, Dixon GH, 1982. Purification and characterization of cytoplasmic protamine messenger ribonucleoprotein particles from rainbow trout testis cells. Biochemistry 21:1869–77

Slaughter GR, Means AR, 1989. Analysis of expression of multiple genes encoding calmodulin during spermatogenesis. Mol Endocrinol 3:1569–78

Strickland S, Huarte J, Belin D, Vassalli A, Rickles RJ, Vassalli JD, 1988. Antisense RNA directed against the 3′ noncoding region prevents dormant mRNA activation in mouse oocytes. Science 241:680–84

Tash JS, Hidaka H, Means AR, 1986. Axokinin phosphorylation by cAMP-dependent protein kinase is sufficient for activation of sperm flagellar motility. J Cell Biol 103:649–55

Tash JS, Means AR, 1983. Cyclic adenosine 3′, 5′ monophosphate, calcium and protein phosphprylation in flagellar motility. Biol Reprod 28:75–104

Uhler MD, Carmichael DF, Lee DC, Chrivia JC, Krebs EG, McKnight GS, 1986a. Isolation of cDNA clones coding for the catalytic subunit of mouse cAMP-dependent protein kinase. Proc Natl Acad Sci USA 83:1300–04

Uhler MD, Chrivia JC, McKnight GS, 1986b. Evidence for a second isoform of the catalytic subunit of cAMP-dependent protein kinase. J Biol Chem 261:15360–63

Vallee RB, DiBartolomeis MJ, Theurkauf WE, 1981. A protein kinase bound to projection portion of MAP 2 (microtubule-associated protein 2). J Cell Biol 90:568–76