Volume 229, number 2, 391–394 FEB 05673 March 1988

A unique mRNA species for a regulatory subunit of cAMP-dependent protein kinase is specifically induced in haploid germ cells

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Received 18 January 1988

Cyclic AMP (cAMP) and its action by way of cAMP-dependent protein kinase is important for sperm motility. Previous studies on germ cells have demonstrated a selective decrease in the amount of type I cAMP-dependent protein kinase during spermatid development, and that type II was the major form present in elongating spermatids and in mature sperm. This would indicate activation of a gene in haploid germ cells, encoding a regulatory subunit of type II protein kinase. However, haploid expression of such a gene has so far not been shown. In the present study we demonstrate high-levelled expression of a unique mRNA species for a specific regulatory subunit of type II cAMP-dependent protein kinase at late stages of spermatogenesis, i.e. during spermatid elongation.

cyclic AMP-dependent protein kinase; Spermatogenesis; Sperm motility

1. INTRODUCTION

In several species, including man, cyclic AMP (cAMP) has been demonstrated to be important for sperm motility [1–3]. Cyclic AMP is known to act via cAMP-dependent protein kinases. The cAMP-dependent protein kinase holoenzyme, consisting of two regulatory (R) and two catalytic (C) subunits, dissociates on binding of two cAMP molecules to each of the R subunits. The activated C subunits then phosphorylate specific substrate proteins on serine and threonine and thereby alter the activity or function of these proteins. Alternatively, cellular effects might be mediated by free R subunits, independent of phosphorylation [4,5]. However, conclusive evidence supporting this possibility is lacking.

Type I- and type II-cAMP-dependent protein kinases are distinguished by their regulatory subunits (RI and RII, respectively). Four different regulatory subunits [RI_α (formerly designated RI) [6], RI_β (Clegg, C., Cadd, G. and McKnight, G.S., in preparation), RII_α (RII_{54}) [7] and RII_β (RII_{51}) [8]] and two different catalytic subunits (C_α [9] and C_β [10,11]) for cAMP-dependent protein kinases have now been identified at the gene/mRNA level. (A nomenclature regarding the various subunits of cAMP-dependent protein kinase in higher eucaryotic cells has not yet been agreed upon. We would like to propose using the designations RI_α, RI_β, RII_α, RII_β, C_α and C_β to make a more uniform and stringent nomenclature for known subunits of cAMP-dependent protein kinase. Subunits denoted α are those first identified and characterized. Furthermore, they seem to be constitutively expressed in most tissues. The β subunits have only recently been identified and in many cases shown to be differently regulated and expressed compared with the corresponding α

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

subunits. This nomenclature will help exchange information about Rs and Cs across higher eucaryotic species.)

We have previously examined the expression of mRNAs for RI_α, RII_β and C_α in different testicular cell types [12]. We found high levels of mRNA for RI_α both in pachytene spermatocytes (premeiotic) and round spermatids (postmeiotic), while RII_β mRNA was detected at low levels in these germ cells. These findings could not account for the previously shown dominance of type II cAMP-dependent protein kinase in postmeiotic germ cells [13,14]. With the recent cloning of a rat cDNA for RII_α [7], we have now been able to investigate the expression of this gene in whole testis during development and in various types of germ cells.

2. MATERIALS AND METHODS

2.1. *Preparing whole testes for age study*

Sprague-Dawley rats of various ages (5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 80 and 100 days of age) were decapitated. Testes were at once dissected free, frozen in liquid N_2 and stored at −75°C for later RNA extraction.

2.2. *Preparation of germinal cell fractions*

Germ cells were isolated from seminiferous tubules of 32 and 44 day old rats, respectively. A cell preparation was obtained by consecutive collagenase-, trypsin-, DNase- and mechanical treatment [12]. The germ cell fractions mentioned above (ES only present in 44 day old rats) were then isolated by the Sta-Put unit gravity sedimentation method using a BSA gradient [15,16] essentially as described by Grootegoed [17]. The germ cells were examined both by phase contrast microscopy and by regular light microscopy after fixation and staining.

The purities were evaluated by counting cells: pachytene spermatocytes (PS), 32 days 90–95%; round spermatids (RST), 32 days 85–90%; PS, 44 days 75–80%; RST, 44 days 65–70% (contamination mostly elongating spermatids (ES)); ES 45–55% (contamination mostly RST).

2.3. *Preparation of total RNA*

RNA extraction from whole testes and from fractionated germ cells was performed as previously described [12] by homogenization in guanidinium isothiocyanate. Total-RNA was isolated by centrifugation through a cesium chloride gradient and purified by phenol/chloroform extractions.

2.4. *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 [12]. 20 μg total-RNA was used in each lane, separated on the gel and transferred to a nylon filter (ICN, Biotrans) by capillary blotting technique. The resulting filter was prehybridized in 50% formamide containing denatured salmon sperm DNA at 42°C, and hybridized under the same conditions using a nick-translated rat cDNA probe (0.7 kb *SalI-BglII* fragment; all within the open reading frame [7]) for RII_α. Washing was performed with 0.1 × SSC [18] at 50°C, and the filter was finally autoradiographed using Hyperfilm MP (Amersham).

2.5. *In-solution hybridization assay*

Total-RNA was isolated from germ cell fractions as described above. The mRNA levels were determined as previously described [9,19], by using an SP6 RNA transcript complementary to the RII_α mRNA. Molecules per cell were calculated by comparison to M13 standards, assuming 6 pg DNA/cell and a 1:1 DNA/total-RNA ratio.

3. RESULTS AND DISCUSSION

In testis obtained from rats less than 40 days of age, we only found the 6.0 kb mRNA similar to that seen in most somatic cells [7,20] (fig.1). The intensity of this band was fainter after 40 days of age indicating that this mRNA is primarily located in the somatic cells of the testis. The apparent decrease is probably due to the rapid proliferation of germ cells occurring at this age, reducing the relative number of somatic cells in the testis. From 40 days of age we found a high level of expression of a smaller sized (2.2 kb) mRNA for RII_α. A similar mRNA species for RII_α was shown specifically in mouse testis (referred to as 2.4 kb) [7], but has not been observed in any other cell or tissue so far examined. Our results suggested the possibility that induction of this small sized mRNA for RII_α occurs specifically at late stages of spermatogenesis. The direct evidence for this is shown in fig.2. Pachytene spermatocytes and round spermatids isolated from 32 day old rats did not express the 2.2 kb mRNA for RII_α. Isolation of pachytene spermatocytes, round spermatids and elongating spermatids from 44 day old rats demonstrated a dramatic expression of the 2.2 kb mRNA in the fraction containing elongating spermatids. Weaker bands corresponding to the 2.2 kb mRNA were also found in the round spermatid and pachytene spermatocyte fractions. However, these messages can be accounted for by contamination of elongating spermatids in the germ cell fractions at this age (estimated purities of germ cell fractions are given in section 2.2).

Taking into account both the developmental studies and the studies performed on isolated cells, we conclude that the 2.2 kb mRNA for RII_α is transcribed in the haploid cells during spermatid

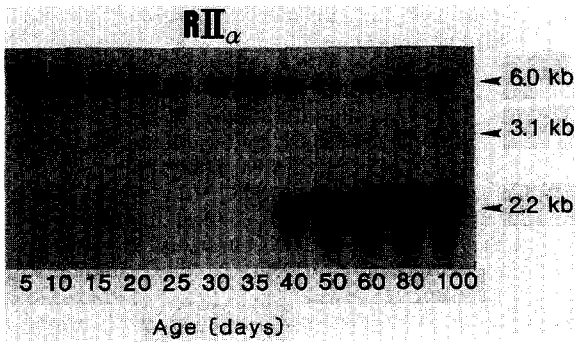

Fig.1. Northern blot showing levels of mRNA for RII$_\alpha$ in testis from rats of different ages. Approximate mRNA sizes are indicated.

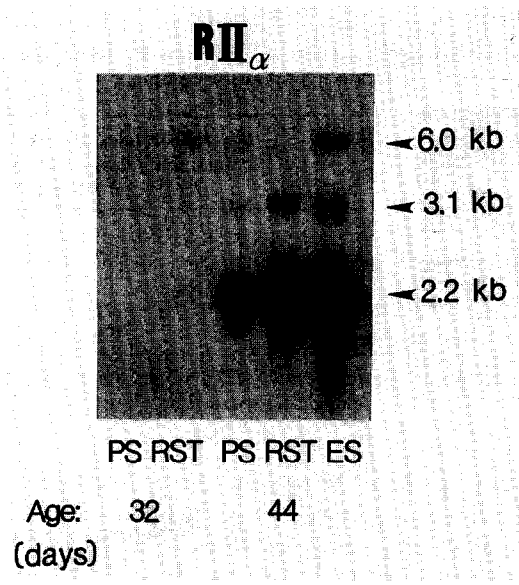

Fig.2. Northern blot showing the levels of RII$_\alpha$ mRNA in different germ cell fractions (PS, pachytene spermatocytes; RST, round spermatids; ES, elongating spermatids) obtained from rats of 32 and 44 days of age. Approximate mRNA sizes are indicated. The above figure represents an overnight exposure (Hyperfilm MP, Amersham).

elongation. A faint 3.1 kb mRNA was also seen both in the developmental studies as well as in the isolated cell fractions. This mRNA, which appeared proportional to the 2.2 kb band, may represent a nuclear precursor but could also arise from the use of another polyadenylation site signal [8].

Table 1 provides quantitative data, from in-solution hybridization assay, relating to fig.2. These data confirm the results from the Northern analysis and support our conclusion that this mRNA is specifically formed during spermatid elongation.

It is likely that the abundant smaller sized mRNA for RII$_\alpha$, appearing during spermatid elongation, may encode the type II protein kinase shown to be selectively enriched in elongating spermatids and spermatozoa [13,14]. This conclusion is difficult to prove, since for the time being it is very hard to distinguish between the subtypes of type II cAMP-dependent protein kinase at the protein level. However, in agreement with previous studies in mouse testis [13], we have obtained results from Western blotting showing that RI decreases and RII increases during germ cell differentiation (not shown).

Based on hybridization data with fragments from the RII$_\alpha$ cDNA clone and with synthetic oligonucleotides (unpublished), we believe that this unique 2.2 kb mRNA for RII$_\alpha$ arises from the same gene as the 6.0 kb mRNA seen in other tissues. In that case, germ cells appear to use a specific polyadenylation signal, which is ignored in other tissues. However, one cannot rule out the possibility that the 2.2 kb mRNA may represent an alternative splicing of the RII$_\alpha$ gene, giving rise to a germ cell specific regulatory subunit.

By this study, RII$_\alpha$ can be included among the haploid-specific genes, as it is obviously turned on at a postmeiotic stage. More precisely we should refer to this unique 2.2 kb mRNA for RII$_\alpha$ as a haploid-specific RNA message. Previously the protamines, a unique α-tubulin [21,22] and the proto-oncogene int-1 [23] have been shown to be specifically expressed in haploid cells.

Recently, axokinin, a 56 kDa protein, was identified [24], and the evidence suggested that axokinin phosphorylation by cAMP-dependent protein kinase is not only required for sperm motility but is sufficient as well [25]. The fact that the RII subunits of cAMP-dependent protein kinases are autophosphorylated at a specific serine residue [14] raises the possibility that RII$_\alpha$ (molecular mass in rat 54 kDa) is identical to axokinin (molecular mass in dog 56 kDa). However, a more likely possibility is that RII$_\alpha$ is essential for effective cAMP-dependent phosphorylation of ax-

Table 1

RII_α mRNA levels in germ cell fractions as measured by in-solution hybridization technique

	Molecules per cell (assuming 6 pg DNA/cell, DNA/total-RNA 1:1)
PS, 32 day old rats	1
RST, 32 day old rats	2
PS, 44 day old rats	29
RST, 44 day old rats	116
ES, 44 day old rats	175

PS, pachytene spermatocytes; RST, round spermatids; ES, elongating spermatids

okinin, perhaps by anchoring the kinase to a subcellular structure where axokinin is readily available for phosphorylation. In brain a cAMP-dependent protein kinase has been shown to be bound to microtubules via a microtubule-associated protein (MAP) [4,26], and a 55 kDa protein seemed a likely candidate for the R subunit of this kinase [26]. This leads to the intriguing hypothesis that RII_α is responsible for anchoring the kinase activity to microtubules of the sperm flagella and thereby promotes sperm motility.

Acknowledgements: This work was supported by the Norwegian Cancer Society (V.H., T.J.), Norwegian Society for Fighting Cancer (V.H., T.J.), Norwegian Research Council for Science and the Humanities (NAVF) (V.H., T.J.), Torsteds grant (V.H., T.J.), Anders Jahres Foundation for the Promotion of Science (V.H., T.J.) and grants from National Institutes of Health (G.S.M.).

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