Expression cloning of a cDNA encoding the type II regulatory subunit of the cAMP-dependent protein kinase

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We report here the isolation and sequence of a cDNA for the type II regulatory subunit of the cAMP-dependent protein kinase (cAMP-PK) from a λgt-11 cDNA library derived from a porcine epithelial cell line (LLC-PK_1). The cDNA was detected by immunological screening using an affinity purified polyclonal antibody for bovine R_{II}. DNA sequence analysis of the 467 bp *Eco*RI insert confirmed the identity of the clone, because the deduced amino acid sequence corresponded to the published sequence for the bovine R_{II} protein. Northern analysis of total RNA from the LLC-PK_1 cells indicated a single mRNA species of about 6.0 kb, probably derived from a single copy gene.

cyclic AMP dependence *Protein kinase* *Regulatory subunit* *Expression cloning* *(LLC-PK_1 cell)*

1. INTRODUCTION

The cAMP-dependent protein kinase (cAMP-PK) plays a central role in the regulation of metabolism and growth of mammalian cells [1], with all available evidence suggesting that it is the sole mediator of cAMP action [1–3]. Based on biochemical analysis the kinase is composed of three different subunits, the catalytic subunit (C) complexed with either type I regulatory (R_I) or type II regulatory subunit (R_{II}) in a heterodimeric conformation (R_2C_2) [4,5]. Recent studies, however, suggest that there are multiple species of the various subunits [6–8]. Two types of R_{II} subunits have been detected in rat ovaries [7], distinguished by peptide maps and by two-dimensional gel electrophoresis of photoaffinity labelled protein. One type corresponds to the neural-specific R_{II} subunit [8,9] which differs immunochemically, and in amino acid sequence [8–11], from the prototype non-neural R_{II} isolated from bovine heart [12,13]. Similarly, the purified bovine heart C subunit can be separated into two distinct forms based on charge differences [14].

In an effort to define the subunits of the cAMP-PK from a clonal cell line and their role in specific regulation of cellular metabolism, we have undertaken the molecular cloning of the kinase. For this study the LLC-PK_1 cell line [15] was used because it produces both type I and type II cAMP-PK [16] and mutant cell lines exist which are affected in cAMP-PK activity [17]. In addition it is a very attractive model system for investigating the role of the cAMP-PK in regulating gene expression because elevation of cAMP levels leads to a 200-fold increase of the transcription of the uPA gene [18].

In this communication we report the molecular cloning of the type II regulatory subunit by use of the λgt-11 expression cloning system. The data show that LLC-PK_1 cells synthesize a type II regulatory subunit that is homologous to the bovine heart R_{II} and not to the neural R_{II}.

2. MATERIALS AND METHODS

2.1. *Cell culture*

The porcine epithelial cell line LLC-PK_1 [15] was

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cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat inactivated fetal calf serum as described [16].

2.2. *Antisera*

Rabbit antisera were raised by subcutaneous injection of purified bovine heart R_{II} conjugated to Keyhole limpet hemocyanin emulsified with complete Freund's adjuvant. The antisera were affinity purified by passage over a column of bovine heart R_{II} covalently linked to Affigel 10. Antisera binding to the immobilized R_{II} were eluted using 6 M urea (in 25 mM Tris-HCl, pH 7.0, 1 mM EDTA) and immediately dialyzed against 20 mM Mes-NaOH, pH 6.9, 150 mM NaCl and 1 mM EDTA. Antisera were of the IgG class, as judged by SDS-polyacrylamide gel electrophoresis.

For screening the expression library, antibodies that cross reacted with bacterial or phage proteins were removed from all antisera by preabsorption with a λgt-11 lysate of *E. coli* strain Y1090.

Antisera to bovine R_{II} were shown by Western blot analysis to cross react with the R_{II} produced by the porcine cell line LLC-PK_1 [16].

2.3. *Construction and screening of the λgt-11 LLC-PK_1 cDNA library*

Poly(A^+) RNA was isolated from LLC-PK_1 cells by the method of Glisin et al. [19] and used to prepare cDNA by the method of Gubler and Hoffman [20]. Following treatment with *Eco*RI methylase and T_4 DNA polymerase, *Eco*RI linkers were added to the cDNA. The cDNA was then size selected by chromatography on Sephacryl S300, coprecipitated with λgt-11 DNA (*Eco*RI-cleaved, bacterial alkaline phosphatase treated), ligated, packaged and amplified on *E. coli* strain Y1088. Approx. 90% of the packaged phage contained inserts, as shown by insertional inactivation of the phage β-galactosidase gene.

The λgt-11 library was screened for antigen producing clones by sequential incubation with anti-R_{II} serum and ^{125}I-labelled protein A as described by Young and Davis [21].

2.4. *Subcloning and nucleotide sequencing*

Inserts from phage that cross reacted with the antiserum were subcloned into M13mp11 for sequence analysis by the dideoxy method of Sanger et al. [22].

2.5. *Blot hybridization analysis*

2.5.1. Northern analysis of LLC-PK_1 RNA

Denatured total RNA was size fractionated by electrophoresis in 1% formaldehyde/agarose gels and transferred to nitrocellulose as described [18]. Blots were hybridized with the *Eco*RI insert of λR_{II} 17 (labelled by random priming [23]) at 65°C in 6 × SSC/2 × Denhardt's solution/0.1% SDS/0.05% $NaPP_i$/herring sperm DNA (100 μg/ml) for 20 h and then washed at 65°C for 2 h in 1 × SSC/0.1% SDS, and then subjected to autoradiography.

2.5.2. Southern blot analysis

Genomic DNA was isolated from LLC-PK_1 cells [24], digested with restriction endonucleases, fractionated on 0.8% agarose gels and transferred to nitrocellulose [25]. The blots were probed with the cDNA insert for R_{II} as described above.

3. RESULTS

3.1. *Expression cloning of R_{II}*

A cDNA library constructed from LLC-PK_1 poly(A^+) RNA in λgt-11 was screened with an affinity purified polyclonal antibody directed against the bovine heart R_{II}. Preliminary experiments demonstrated that the antibody preparation binds porcine R_{II} as efficiently as bovine R_{II}. From the initial screening of approx. 10^6 phage plaques 2 positives (λR_{II} 21 and λR_{II} 17) were obtained, rescreened and plaque purified. The positives represented two cDNA classes of 119 and 467 bp, respectively. Cross hybridization between the two cDNAs was not observed.

3.2. *Sequence analysis of R_{II} cDNA*

Sequence analysis of the two cDNA inserts was undertaken using the dideoxy method of Sanger et al. The results showed that the 467 bp insert of λR_{II} 17 coded for type II R subunit (fig.1), corresponding to amino acids 55 (Ser) to 209 (Tyr) of the protein sequence for the bovine R_{II} [12]. Examination of the region around the autophosphorylation site (Ser^{95}) shows that the sequence is similar to the heart isotype and not to the neural isotype. Both isotypes of R_{II} have been sequenced at the protein level in this region and show 4 distinctive amino acid differences within a 15 amino acid stretch [10,11].

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 55                  Phe Asp      Gly Ala              Ala          Val
TCC GGC TCC CAG GAT CTA GAG CCC AGC TCT GGC CTT GTC ACC GAC GCG ATC GCG GAC AGC
Ser Gly Ser Gln Asp Leu Glu Pro Ser Ser Gly Leu Val Thr Asp Ala Ile Ala Asp Ser

 75              Glu                                  Gly
GAG TCG GAG GAC GAC GAG GAC TTG GAC GTT CCA ATT CCT AGC AGA TTT GAT CGG CGA GTA
Glu Ser Glu Asp Asp Glu Asp Leu Asp Val Pro Ile Pro Ser Arg Phe Asp Arg Arg Val

 95
TCA GTC TGT GCT GAG ACC TAT AAC CCT GAT GAG GAA GAG GAA GAT ACT GAT CCA AGG GTG
Ser Val Cys Ala Glu Thr Tyr Asn Pro Asp Glu Glu Glu Glu Asp Thr Asp Pro Arg Val

115
ATT CAC CCT AAA ACT GAT CAA CAA AGA TGC AGA CTT CAA GAA GCT TGC AAA GAT ATT CTT
Ile His Pro Lys Thr Asp Gln Gln Arg Cys Arg Leu Gln Glu Ala Cys Lys Asp Ile Leu

135                     Pro
CTT TTC AAA AAT CTT GAT CAG GAA CAA CTT TCT CAA GTC CTC GAT GCT ATG TTT GAA AGG
Leu Phe Lys Asn Leu Asp Gln Glu Gln Leu Ser Gln Val Leu Asp Ala Met Phe Glu Arg

155
ACA GTC AAA GTT GAT GAG CAT GTC ATT GAC CAA AGA GAT GAT GGA GAC AAC TTT TAT GTT
Thr Val Lys Val Asp Glu His Val Ile Asp Gln Arg Asp Asp Gly Asp Asn Phe Tyr Val

175
ATT GAA CGG GGA ACC TAT GAT ATT TTA GTA ACA AAA GAT AAT CAA ACC CGC TCT GTT GGT
Ile Glu Arg Gly Thr Tyr Asp Ile Leu Val Thr Lys Asp Asn Gln Thr Arg Ser Val Gly

195                                                  209
CAG TAT GAC AAC CAT GGC AGT TTT GGA GAA CTA GCT CTG ATG TAC AG
Gln Tyr Asp Asn His Gly Ser Phe Gly Glu Leu Ala Leu Met Tyr
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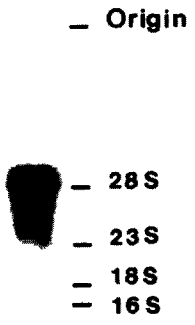
Fig.1. DNA sequence and deduced amino acid sequence of the porcine type II regulatory subunit cDNA. The 467 bp insert was sequenced by the dideoxy method. The amino acids above the DNA sequence correspond to the sequence for the bovine R_{II} [12].



The deduced amino acid sequence of λR_{II} 21 did not appear to code for R_{II}. However, a pentapeptide sequence -Phe-Asp-Pro-Gly-Ala- of R_{II} was found which could represent an epitope recognised by the anti-R_{II} antisera.

3.3. *Northern analysis*

Total RNA from LLC-PK_1 cells was analyzed by Northern blot hybridization and shown to contain a single high molecular mass mRNA for the R_{II} (fig.2). The R_{II} mRNA was calculated to be approx. 6 kb in length suggesting that the mRNA contains approx. 4.5 kb of noncoding sequences.

Fig.2. Hybridization of the porcine cDNA for the type II regulatory subunit to Northern blots of total RNA (20 μg) from LLC-PK_1 cells. The RNA size markers are porcine and *E. coli* ribosomal RNA.

3.4. *Southern analysis*

The probe for the porcine R_{II} was used to carry out Southern analysis of genomic DNA isolated from LLC-PK_1 cells. The results (not shown) indicate that 1 or possibly 2 genes code for the R_{II} in LLC-PK_1 cells.

4. DISCUSSION

The availability of a cDNA for the type II regulatory subunit will enable us to investigate the heterogeneity of R_{II} [6–8]. Recent evidence [7] has been presented for 2 different isotypes of the R_{II} in ovarian tissue, which probably represent at least the bovine heart and the brain isotypes. Whether these proteins are synthesized by specific cell types or are both synthesized by a single cell type remains to be established. Our evidence indicates a single mRNA species in LLC-PK_1 cells.

From the analysis of the available sequence data the R_{II} synthesized by LLC-PK_1 cells would appear to be very similar to the bovine heart isotype [10–12]. Of the 155 amino acids for which sequence was available, there were only 9 sequence differences between porcine and bovine proteins, 4 of which were conservative changes. Such a high level of homology (94.2%) has been observed also for bovine and porcine R_I [26] and C subunits (Adavani, S.R. et al., unpublished) of cAMP-PK, implying a central functional role of the kinase in regulation of cellular processes.

The availability of the cDNA for the R_{II} will enable us to isolate and characterize the gene(s) which codes for this protein. We have isolated the porcine gene encoding the type I R subunit [26] and it will be of considerable interest to compare the 5′-promoter regions and the intron/exon organisation, of these two closely related genes. DNA sequence analysis may demonstrate a common ancestral origin for the two.

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