Isolation of a Complementary DNA Encoding the Catalytic Subunit of Protein Kinase A and Studies on the Expression of This Sequence in Rat Hepatomas and Regenerating Liver

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ABSTRACT

A complementary DNA (cDNA) clone (B4) encoding the catalytic subunit of a cAMP-dependent protein kinase (PKAc) was isolated from a rat brain cDNA library, using a synthetic oligonucleotide probe whose sequence was based on the known amino acid sequence of a bovine cardiac PKAc. Sequence analysis of this clone revealed a region of 1002 nucleotides which encodes a protein that is 92% homologous to amino acids 17-350 of the bovine cardiac PKAc protein. This clone lacks coding sequences for amino acids 1-16 of the latter protein. Nevertheless, it provided a useful probe to analyze expression of the related gene in a variety of systems. Northern blot analyses using a 32P-labeled probe prepared from a 0.6-kilobase PstI fragment of clone B4 revealed an abundant 4.6-kilobase band in rat brain RNA and lesser amounts of this 4.6-kilobase RNA in rat heart and liver. A 4.6-kilobase RNA was also detected in RNA samples obtained from mouse fibroblasts. This probe also detected homologous RNA in a variety of nonrodent species. In subsequent experiments, this cDNA was used as a probe to elucidate the role of PKAc in post-surgical hepatic regeneration and diethylnitrosamine-induced hepatomas in the rat. These experiments revealed that, following partial hepatectomy, PKAc mRNA is decreased 3-fold by 12 h, returning to normal by 72 h; hepatomas showed no consistent pattern of change in PKAc mRNA levels as compared to controls. Our results indicate that this cDNA encodes an isoform of PKAc which is distinct from PKAc-α isolated by Uhler et al. (Proc. Natl. Acad. Sci. USA, 83: 1300-1304, 1986) but highly homologous to PKAc-β isolated by Showers and Maurer (J. Biol. Chem., 261: 16288-16291, 1986), that depression of cAMP-dependent protein phosphorylation may be an important mechanism in the regeneration of mature rat liver but is not a consistent alteration in chemically induced hepatoma, and that this cDNA is useful as a probe for the study of the role of PKAc gene expression in growth control, particularly in rodent species.

INTRODUCTION

The importance of protein phosphorylation in the regulation of cellular growth and metabolism is widely recognized and has been a major focus of research efforts in recent years (for review see Ref. 1). Among the principal protein kinases which regulate cellular processes is the cAMP-dependent protein kinase (PKA). The PKA holoenzyme is a heterotrimer composed of two regulatory subunits which bind to and inhibit two catalytic subunits. PKA is activated when 4 molecules of cAMP, formed in the cell in response to various tissue-specific extracellular signals, bind to the regulatory subunit dimer with high affinity, causing it to release two active molecules of catalytic subunit, which then phosphorylate specific intracellular targets (2, 3).

Notwithstanding suggestions that free regulatory subunits have a biological function independent of the titration of the catalytic subunit (4), the principal effect of cAMP on cells appears to be the activation of PKAc. It is therefore of interest to study PKAc directly as a means of approaching cAMP-dependent phenomena. Shoji et al. (5) deduced the amino acid sequence of this enzyme isolated from bovine heart, finding it to be composed of 350 residues; other characteristics of this protein, such as phosphorylation sites (6) and N-terminal tetradecanoate capping (7), have been extensively studied. More recently, several groups have isolated cDNA clones encoding this protein from murine, bovine, and human cDNA libraries (8-11). Significantly, these studies have revealed that, in parallel with recent work on PKC (for review see Refs. 12 and 13), there exists a family of genes encoding cAMP-dependent protein kinases, which differ in their primary amino acid sequences, their message size, and their tissue specificity. In this paper we describe the isolation of a clone from a rat brain cDNA library which encodes a PKAc similar to the β-form previously isolated from murine and bovine sources and examine its expression in various tissues and species. It was of particular interest to use this cDNA as a probe to explore the role of PKAc gene expression in cellular proliferation in hepatic regeneration and hepatic neoplasia in the rat. Experimental evidence has implicated cAMP, and by extension cAMP-dependent protein phosphorylation, as an important negative regulator of cell growth. Early work (14) demonstrated that cAMP, when added to transformed cells in culture, causes a partial reversion of the transformed phenotype. Subsequent studies by numerous investigators working in a variety of systems (15, 16) have explored this idea, with considerable variability of findings. In hepatoma cells, AMP-associated receptors (e.g., adrenergic, gluconeog, and prostaglandin) have been shown to be decreased, as were overall cAMP levels as compared to controls (17, 18). In contrast, in liver cells which have emerged from quiescence following partial hepatectomy, many studies have demonstrated transient increases in intracellular cAMP levels, but an overall decrease in adenylate cyclase activity with time (19), and elevated levels of β-adrenergic receptors (20).

In order to explore this issue at the level of expression of the gene encoding the effector molecule of cAMP, PKAc, we have used the cDNA whose isolation is described here as a probe to measure levels of PKAc gene expression in regenerating rat liver following partial hepatectomy and in a series of primary rat hepatomas. Our results suggest that cellular proliferation in hepatomas and in hepatic regeneration may be mechanistically different, the latter being associated with a fall in PKAc levels.

MATERIALS AND METHODS

Preparation of the Probe. The 54-base oligonucleotide probe (Fig. 1) and a 19-base oligonucleotide primer which hybridized to the nondegenerate 3' end of the probe were synthesized on an automated DNA
PKAc IN RAT HEPATOMAS AND REGENERATING RAT LIVER

Fig. 1. The open reading frame of the cDNA clone B4. Upper lines indicate the nucleotide sequence; the corresponding amino acid sequence is shown below this. Differences between the translated nucleic acid sequence of B4 and that of a bovine cardiac PKAc (5) are underlined. Regions of homology of amino acid sequences to a protein kinase C (12) are in boxes. The regions of B4 corresponding to the synthetic probe used to isolate it are double-underlined.

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synthesizer (Applied Biosystems 380A). The oligonucleotide probe was designed, using probable codon usage tables (21), to hybridize to a nucleic acid corresponding to amino acids 217–235 of a bovine cardiac PKAc whose amino acid sequence had been determined by Shoji et al. (5). Yields of the synthesized oligonucleotide were analyzed using a trityl ion colorimetric assay, as described by the manufacturer. The primer was purified on a 20% acrylamide-50% urea gel, and the probe was purified using a C18 reverse phase high performance liquid chromatography column run with a continuous 0–40% acetonitrile gradient.

Three pmol of the probe in a volume of 3 μl were hybridized to 15 pmol of the primer in a volume of 1.1 μl, together with 1.5 μl of 10× reverse transcriptase buffer (22), at 55°C for 2 h. This mixture was then cooled briefly and combined with 2 μl each of 32P-labeled and unlabeled dATP, dCTP, dGTP, and dTTP, 1 μl of 10× reverse transcriptase buffer, 0.5 μl water, and 1 μl avian myeloblastosis virus (AMV) reverse transcriptase (Life Sciences). This mixture was incubated at 37°C for 1 h, and the resulting 32P-labeled probe was used to screen a cDNA library, as described below.
Screening of cDNA Libraries. λgt10 CH1 rat brain cDNA libraries (library A, courtesy of R. Axel, Columbia University; library B, courtesy of J. Brosius, Mount Sinai School of Medicine) were plated at a density of 5 × 10⁴ clones/15-cm plate, with a total number of plaques of 6 × 10⁶. These plates were blotted with Hybond-N nylon membranes (Amerham Corp., Arlington Heights, IL) and processed according to the manufacturer’s protocol. Filters from library A were prehybridized in a solution of 6x SSC, 5x Denhardt’s solution, 50 mM Na₂PO₄, 2% sodium dodecyl sulfate, 20% formamide, and 2.5 μg denatured salmon sperm DNA, in a total volume of 125 ml, for 2 h at 42°C. The filters were then hybridized in a solution of identical composition but with the addition of the above-described ³²P-labeled probe, at 42°C for 24 h, in a total volume of 50 ml. The filters were then washed to a stringency of 0.2x SSC. Positively hybridizing clones were plaque purified by repeating at lower density and screening as before. Single hybridizing plaques were stored in SM buffer containing 0.1% gelatin (22) and a trace of chloroform. Filters from library B were prepared and positive clones were picked and purified in a manner identical to that used with library A.

DNA Sequencing and Vectors. DNA fragments obtained from the above-described clones were subcloned into two Gemini series vectors (pGEMI or pGEM2) (Promega Biotech), for restriction mapping and chemical sequencing (23), or into bacteriophage m13 vectors (mp18 and mp19), for dye-oxy chain-termination sequencing (24, 25). Plasmids were prepared by the alkaline lysis method and banded in a CsCl gradient (22). Restriction mapping of DNA was performed with enzymes from either New England Biolabs or Boeringer Mannheim Biochemicals. Sequencing gels were 0.4 mm and contained 6% acrylamide: bis (19:1) and 8 M urea.

Liver Tissue Preparation. All livers were from Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) and were from females unless otherwise indicated. Liver tumors were initiated neonatally by a single i.p. injection of diethylnitrosamine and, following weaning, rats were placed on a diet containing phenobarbital (0.025–0.1% of diet) to promote the formation of tumors. The rats were killed by cervical dislocation. The livers were removed and were immediately excised and frozen in liquid nitrogen. Control livers were obtained from 200-day-old female Sprague-Dawley rats obtained from Charles River Laboratories. The pathology of the samples was verified by routine histology. Additional details have been previously described (26).

Liver regeneration experiments were carried out by placing female Sprague-Dawley rats on a diet containing either 30% casein (control) or 0.05% phenobarbital plus casein for 16 days prior to partial hepatectomy. Cervical dislocation was performed on three rats each at 12, 24, 48, 72, and 168 h following surgery. Tissues were immediately frozen in liquid nitrogen and stored at −70°C until processing. Control livers for each animal were those tissues removed at the time of hepatectomy.

Northern Blot Analyses. Total RNA was isolated from various cell lines and tissues using a previously described extraction procedure (27). Equivalent amounts of RNA (10 μg/lane) were loaded onto 1% agarose gels containing 6% formaldehyde, electrophoresed, and blotted onto nylon membranes, as described by the manufacturer (Amerham, Arlington Heights, IL). The studies on regenerating rat liver and rat hepatomas (Figs. 4 and 5) employed polyadenylated RNA (4 μg/lane), which was isolated as previously described (26). A ³²P-labeled probe of the Psrl fragment of clone B4 was prepared by nick translation (28), hybridized at 42°C to the nylon filters containing rat RNAs in buffer containing 50% formamide, and washed to a stringency of 0.1x SSC at 65°C, according to the manufacturer’s protocol (Amerham). Filters containing RNAs from other species were hybridized to the same probe at 37°C in buffer containing 40% formamide and washed to a stringency of 2x SSC at 50°C. Molecular weights of the bands were based on the 18S and 28S ribosomal markers. All lanes contained equivalent amounts of RNA, based on ethidium bromide staining.

RESULTS

An 18-amino acid peptide sequence (residues 217–235) present in bovine cardiac PKAc (5) was used to design a complementary 54-base oligonucleotide probe to detect a gene encoding this peptide sequence (see “Materials and Methods”). This synthetic oligonucleotide differed from the corresponding region of the cDNA which we subsequently isolated (see below) at only five positions other than those anticipated on the basis of codon preference tables.

Two λgt10 cDNA libraries derived from female CH1 rat brain were used in these studies. Library A (provided by R. Axel) had an estimated complexity of 1 × 10⁶ clones, and library B (provided by J. Brosius) had a complexity of 6 × 10⁶ clones. Library A failed to yield a full length PKAc cDNA clone. It did, however, provide a clone that contained a 600-base pair Psrl-Psrl fragment encoding a polypeptide highly homologous to a portion of bovine PKAc. This Psrl-Psrl fragment was used to screen library B for a full length cDNA clone. Fourteen recombinant plaques hybridized to this probe. Three of these appeared to contain the same insert, which was approximately 3 kilobases in length. The most abundant of these clones, clone B4, was subcloned into plasmid and phage vectors, and a detailed sequence analysis of clone B4 was performed (see “Materials and Methods”). The sequence obtained revealed a 1002-nucleotide open reading frame followed by a stop codon (Fig. 1). Also indicated in Fig. 1 is the extensive homology of the predicted amino acid sequence encoded by B4 to the previously reported amino acid sequence of bovine cardiac PKAc. Despite this remarkable homology (92%), the B4 cDNA sequence is slightly truncated at its 5’ end, since it lacks the 5’ terminal 51 nucleotides that would encode a 17-amino acid sequence present at the amino terminal end of the bovine PKAc sequence. Southern blot analyses are consistent with a single copy of the corresponding gene in the rat genome (data not shown).

We examined the expression of RNAs homologous to rat PKAc in various tissues, using B4 as a probe in Northern blot analysis. Fig. 2 indicates that we detected a 4.6-kilobase band in RNA samples isolated from various rat tissues (brain, heart, and liver), the K16 and K22 rat liver epithelial cell lines, the Rat6 fibroblast cell line, human esophagus, the human GM6167 fibroblast cell line, two samples of mouse liver, and the Chinese hamster ovary cell lines CHO 10001 and CHO 10260 (a variant of CHO 10001, which is deficient in PKA.}

Fig. 2. Northern blot analysis of RNA samples from various tissues using the B4 sequence as a probe. Each lane contained 10 μg of total RNA from rat brain (lane 1), heart (lane 2), and liver (lane 3); rat liver epithelial cell lines K16 (lane 4) and K22 (lane 5); rat6 fibroblasts (lane 6); human esophagus (lane 7); human GM6167 fibroblasts (lane 8); mouse liver (lanes 9 and 10); and the Chinese hamster ovary cell lines 10001 and 10260 (lanes 11 and 12). Molecular weights are indicated at the right. For additional details see “Materials and Methods.”
activity) (29). The relative abundance of this RNA species in the rat brain, heart, and liver samples (which contain equivalent amounts of RNA/lane) corresponds approximately to the reported relative tissue abundance of PKAc protein in these tissues (30).

It was also of interest to compare our rat B4 clone to the murine PKAc-α and PKAc-β clones isolated by other investigators (8, 9, 11). Fig. 3 indicates a Northern blot of mouse liver RNA hybridized to nick-translated probes prepared from the B4 clone (Fig. 3A), the murine PKAc-α (8) (Fig. 3B), or the murine PKAc-β (11) (Fig. 3C). As is apparent in the figure, both the rat B4 and the murine PKAc-β probes detected a prominent 4.6-kilobase band, whereas the murine PKAc-α probe detected a fainter lower molecular weight band of approximately 2.3 kilobases. The presence of low molecular weight RNA bands in tissues from mouse liver, CHO 10001 cells, and human brain, using B4 as a probe (Fig. 2), and in mouse liver, using murine PKAc-β as a probe (Fig. 3C), may represent cross-hybridization to other isoforms of PKAc; alternatively, they may indicate RNA degradation products of the intact higher molecular weight form. This aspect requires further study.

Fig. 4A depicts the densitometric data obtained from Northern blot analysis of the time course of expression of PKAc in regenerating rat liver. RNA was collected from triplicate animals at time points of 12, 24, 48, 72, and 168 h after partial hepatectomy from phenobarbital-fed and casein control rats, as described in “Materials and Methods.” Error bars represent 1 SE. The level of message expression at the 168-h time point is unchanged from 72 h and is not shown. It is apparent from the figure that liver regeneration is associated with a 3-fold reduction in the abundance of PKAc mRNA at 12 h following partial hepatectomy. This reduction is statistically significant based on calculations of the SE of the three densitometric data points. These data also suggest that the fall in PKAc message levels returns to baseline by 72 h. Furthermore, we found that these changes were not affected by the administration of phenobarbital, a known promoter of liver tumor induction in rats (26). Fig. 4B is an example of the Northern blot analysis from which the densitometric data were obtained. Shown are the 12-h time point RNA samples obtained from the partially hepatectomized and control animals. Since the rats used in this study were outbred, it was necessary to use the liver resected at the time of hepatectomy as an internal control for each regenerating liver removed at different times following hepatectomy. The markedly and consistently decreased PKAc message expression is readily apparent in the figure.

Fig. 5 shows the Northern blot hybridization analysis of the expression of PKAc mRNA in rat liver tumors, which were induced as described in “Materials and Methods.” As apparent from the figure, there was no consistent pattern of the mean induction or reduction of expression of this gene in liver tumors.
when compared to the normal livers of adult control rats, nor were there significant differences in the expression of this gene in hepatocellular carcinoma versus adenomas. Adenomas in many male rats showed increased levels of PKAc gene expression, as compared to controls, and several of the adenomas in female rats displayed minor decreases in PKAc mRNA levels, these alterations, however, were inconsistent within these groups. These conclusions were confirmed by densitometric scanning of the autoradiograms. Ethidium bromide staining of ribosomal markers indicated that equal amounts of RNA were loaded in each lane.

DISCUSSION

The present studies describe the isolation from a rat brain cDNA library of a cDNA clone, designated B4, that contains an open reading frame of 1002 nucleotides which encodes a protein that is 94% homologous to the amino acid sequence of a bovine cardiac PKAc (5). The predicted amino acid sequence contains regions which are conserved among several different protein kinases (31), including the catalytic regions of PKC (13) and the cGMP-dependent protein kinase (32). A comparison with the amino acid sequence of bovine cardiac PKC strongly suggests that the B4 clone is truncated by at least 51 nucleotides at its 5' end. Presumably this occurred during construction of the cDNA library, since the 5' end of the open reading frame of B4 contains an EcoRI site, but this requires further study.

During the course of our studies, other laboratories have reported the isolation from various sources of cDNAs for PKAc (8, 9, 11). It is of interest, therefore, to compare our results with theirs. Our Northern blot analyses, using a PstI-PstI fragment of clone B4 as a probe, detected a 4.6-kilobase RNA species in both rat and mouse cells (Figs. 2 and 3). Uhler et al. (8, 11) have isolated two PKAc cDNAs (designated c-α and c-β) and found that the c-α clone hybridizes to a 2.4-kilobase species and the c-β clone hybridizes to a 4.3-kilobase species of murine RNA. The homology of our B4 clone to their c-α and c-β clones, at the nucleic acid level, is about 76 and 92%, respectively; the homology of B4 to the bovine c-β of Showers and Maurer is 86%. Using probes generously supplied to us by Dr. G. Stanley McKnight, University of Washington, we have found that our rat clone, B4, detects a 4.6-kilobase RNA species that appears to be the same as that detected by murine PKAc-β, whereas murine PKAc-α detects a 2.3-kilobase RNA (Fig. 3). These results, together with the above-described sequence homologies, suggest that our rat B4 clone and the murine PKAc-β are species variants of the same isoytype of PKAc. The rodent genome, therefore, encodes at least two distinct PKAc sequences. A human form of PKAc-α (10) and two isoforms of porcine PKAc (33) have also recently been cloned. The existence of multiple forms of PKAc, coupled with the existence of multiple forms of the regulatory subunit of PKA (34, 35), may provide mechanisms by which cAMP can exert complex and different effects in different tissues. It remains to be determined whether the individual forms of PKAc differ with respect to their tissue-specific expression and regulation and the protein substrates that they phosphorylate. It is of interest that another protein kinase, PPKC, also belongs to a multigene family and that there is differential expression of the individual genes in different tissues (12, 13).

The usefulness of our clone as a molecular probe to study levels of expression of the gene encoding PKAc was well illustrated by the liver regeneration and hepatoma experiments. The liver regeneration studies revealed a significant decrease in the abundance of PKAc mRNA levels in rat liver 12 h following partial hepatectomy. This change was independent of whether the rats were fed the liver tumor promoter phenobarbital. These results suggest that decreased expression of this gene may play a role in liver regeneration by modulating the level of cAMP-dependent protein phosphorylation in these cells; furthermore, they imply that the mechanism of hepatocyte promotion by phenobarbital is not by alteration in PKAc gene expression. This putative role of a reduction in PKAc expression in liver regeneration is emphasized by the finding (36) that overall levels of protein synthesis and RNA synthesis are increased at 7 and 16 h following partial hepatectomy, despite our finding of a decrease in the level of a specific PKAc mRNA during that time period. These decreases in PKAc may be mechanistically important in enabling mature hepatocytes to emerge from quiescence and restore hepatic organ mass to normal levels. In contrast to the findings in regenerating liver, we did not find a consistent change in the mean level of PKAc mRNA in a series of carcinogen-induced rat hepatomas, although increases in PKAc message levels in several individual adenomas in male rats and, to a lesser extent, decreases in levels in some adenomas in female rats were seen. The significance of this heterogeneity is not known.

In summary, we have isolated a cDNA which encodes the catalytic subunit of PKAc but which is slightly truncated at the 5' end. Attempts to isolate this fragment are in progress. Nevertheless, this cDNA has been shown to be useful as a probe in the study of the expression of the PKAc gene in two distinct circumstances of altered growth, controlled hepatic regeneration and dysregulated growth of liver tumors. In addition, the present studies provide further evidence that PKAc, like PKC (13), represents a multigene family, which may help to explain the multiple roles these protein kinases play in the regulation of gene expression and growth control.

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Isolation of a Complementary DNA Encoding the Catalytic Subunit of Protein Kinase A and Studies on the Expression of This Sequence in Rat Hepatomas and Regenerating Liver

Jeffrey S. Roth, Ling-Ling Hsieh, Carl Peraino, et al.


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