Isolation and characterization of a mammalian gene encoding a high-affinity cAMP phosphodiesterase

(neurobiology/oncogenes/yeast expression vectors/polymerase chain reaction)

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ABSTRACT A rat brain cDNA library has been constructed in a Saccharomyces cerevisiae expression vector and used to isolate genes that can function in yeast to suppress the phenotypic effects of ras2val19, a mutant form of the RAS2 gene analogous to an oncogenic mutant of the human Hras gene. One cDNA, DPDE, was cloned and its genetic and biochemical properties were characterized. A DPDE product would share 80% amino acid sequence identity with the Drosophila melanogaster dune-encoded protein over an extended region. We have shown that the DPDE protein is a high-affinity cAMP-specific phosphodiesterase.

The yeast Saccharomyces cerevisiae encodes two genes, RAS1 and RAS2, that have structural and functional homology with mammalian ras oncogenes (1-4). When an activated form of the RAS2 gene (RAS2val19) is present, yeast cells fail to synthesize glycerol, are unable to arrest in G1, are intolerant of nutrient starvation, and are acutely sensitive to heat shock (5, 6). These phenotypes, collectively referred to as loss of growth control, are primarily the result of overexpression or uncontrolled activation of the cAMP effector pathway via adenyl cyclase (2, 5, 7, 8).

We have previously reported the isolation of two yeast genes, PDE1 and PDE2, the low- and high-affinity cAMP phosphodiesterase-encoding genes, respectively, by their ability to suppress the heat shock sensitivity in yeast cells harboring an activated RAS2val19 gene (6, 9). We now report the use of a rat brain cDNA library to clone a mammalian cDNA that is able to complement the loss of growth control associated with this activated RAS2 gene in yeast. The gene, DPDE (dunce-like phosphodiesterase), encodes a high-affinity cAMP phosphodiesterase that is highly homologous to the cAMP phosphodiesterase encoded by the dune locus of Drosophila melanogaster. D. melanogaster with mutations in dune have learning and memory defects (10, 11).

MATERIALS AND METHODS

Strains, Media, Transformations, and Heat Shock. Escherichia coli strain HB101 was used for plasmid propagation and isolation, and strain SC51 (Stratagene) was used for transformation and maintenance of the cDNA library (12, 13). S. cerevisiae strains TK161-R2V (MAta leu2 his3 ura3 trpl ade8 can1 ras2 (val19) (5) and 10DAB (MAta leu2 his3 ura3 ade8 pde1::ADE8 pde2::URA3 rasl::HIS3) were used. 10DAB was created from a segregant of a diploid strain produced by mating TS-1 (14) and DJ23-3C (15). The segregant (MAta leu2 his3 ura3 ade8 pde1::LEU2 pde2::URA3 rasl::HIS3) was subsequently transformed with the 5.4-kilobase-pair (kbp) Xba I pde1::ADE8 fragment of pYT19DAB to yield strain 10DAB. Yeast cells were grown in either rich medium (YPD, yeast extract/peptone/dextrose) or synthetic medium with appropriate auxotrophic supplements (SC) (16). Transformation of yeast cells was performed with lithium acetate (17). Heat shock experiments were performed by replica plating onto preheated SC plates that were maintained at 55°C for 10 min, allowed to cool, and incubated at 30°C for 24-48 hr. Segregation analysis was performed by growing yeast transformants in YPD for 2-3 days, plating onto YPD plates, and replica plating onto YPD, SC-leucine (plasmid selection), and YPD heat shock plates.

Plasmids, DNA Manipulations, and Sequencing. Plasmid DNA from individual E. coli colonies was purified by standard procedures (18, 19). Extrachromosomal DNA was isolated from yeast as described (9). The plasmid pYT19DAB was constructed from pYT19 (9) by first deleting PDEI sequences between the Smal I and Bal I restriction sites to yield pYT19D. The 4-kbp BamHI fragment of the Ade8 gene was then inserted into the BamHI site of pYT19D to yield pYT19DAB. The cloning vector pADNS is based on the plasmid pAD1 previously described (20). pADNS consists of a 2.2-kbp Bgl II/Hpa I fragment containing the S. cerevisiae LEU2 gene from YEp213 (21), a 1.6-kbp Hpa I/HindIII fragment of the S. cerevisiae 2-μm plasmid containing the origin of replication, and a 2.1-kbp Ssp I/EcoRI fragment containing the ampicillin-resistance gene from the plasmid pUC18. It also contains a 1.5-kbp BamHI/HindIII fragment of the modified S. cerevisiae alcohol dehydrogenase (ADHI) promoter (22, 23) and a 0.6-kbp HindIII/BamHI fragment containing the ADHI terminator sequences. The promoter and terminator sequences are separated by a polylinker that contains the restriction endonuclease sites Not I, Sac II, and Sfi I between the existing HindIII and Sac I sites. The oligonucleotides used to create these sites were 5'-GGCCAAAAAGCGGGCGCGCA and 5'-TCGACCGCTTCTCGCGGCCTCGCTCG. The plasmid pADP is a pADNS-derived plasmid containing the 2.17-kb DPD cDNA insert.

Sequencing was performed by the dideoxynucleotide chain-termination method (24, 25). GENALIGN was used to align the DPD and dune sequences (GENALIGN is a copyrighted software product of IntelliGenetics; developed by Hugo Martinez). RNA was purified from Sprague-Dawley rat brains by published procedures (26-28). cDNAs were ligated to the Not I linker oligonucleotides 5'-AAACCGGCCGC-3' and 5'-GCGGGCGTGTC-3'. The cDNAs were cleaved with Not I and cloned into the Not I site of pADNS by standard procedures.

Polymerase chain reactions (PCR) were carried out in a thermocycler (Perkin-Elmer/Cetus) using a modification of

Abbreviation: PCR, polymerase chain reaction.

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1The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04563).
published procedures (29). Reaction mixtures contained template DNA (1 ng of cloned DNA or 1 μg of total first-strand cDNA), 25 pmol of oligonucleotide primers, 200 μM deoxyribonucleotide triphosphates, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl₂, and 0.01% (wt/vol) gelatin. The oligonucleotide primers used, as designated in Fig. 3, were as follows: A, 5'-CACCTGTCGAAACACTT-3'; B, 5'-ATGGAGACCGCTGGAGGAA-3'; C, 5'-ATAGCCGCAATCAGA-3'; D, 5'-TACCAAGATGTATTCCC-3'; E, 5'-TGTTCGAGCAGACTTG-3'; F, 5'-GCACACAGTTGGCGAGC-3'. The numbers indicate position coordinates in Fig. 2. Primers C, E, and F are noncoding strand sequences. Thirty cycles (1.5 min at 94°C, 3 min at 55°C, and 7 min at 72°C) were performed and the reaction products were analyzed by polyacrylamide gel electrophoresis.

**Phosphodiesterase Assays.** Yeast cells were grown at 30°C for 36 hr in 1-liter cultures of synthetic medium (SC-leucine). Cells were harvested and washed with buffer C (20 mM Mes/0.1 mM MgCl₂/0.1 mM EGTA/1 mM 2-mercaptoethanol), were resuspended in 30 ml of buffer C with 50 μl of 1 M phenylmethylsulfonyl fluoride, and were disrupted with a French press. The extracts were centrifuged at 1600 × g for 10 min and the supernatants were spun at 18,000 × g for 90 min (4°C). The supernatant was assayed for phosphodiesterase activity (6, 9). All the reaction mixtures contained Tris-HCl (pH 7.5) (100 mM), cell extract (50 μg of protein per ml), 5'-nuclease (20 ng/ml; Sigma), and MgCl₂ (10 mM) (unless otherwise stated) and the indicated cyclic nucleotide concentrations. Assays for cGMP hydrolysis used 1.5 μM cGMP. Inhibition studies used 5 μM cAMP in the presence of various amounts of cGMP up to 500 μM. [³H]cAMP and [³H]cGMP were from NEN. Reaction mixtures were incubated for 10 min at 30°C and stopped with 5× stop solution (250 mM EDTA/25 mM AMP/100 mM cAMP).

**RESULTS**

**A Mammalian Gene That Can Revert the Heat Shock Sensitivity of RAS2<sup>val19</sup> Yeast.** We have previously described the isolation of several yeast genes that when overexpressed on extrachromosomal yeast vectors are capable of suppressing the heat shock sensitivity exhibited by the RAS2<sup>val19</sup>, expressing strain TK161-R2V (6, 9). We have now used the same selection to isolate mammalian genes that can function in yeast to render RAS2<sup>val19</sup> cells resistant to heat shock. A rat brain cDNA library was produced and cloned into the yeast expression vector pADNS (Fig. 1). cDNAs were ligated to Not I linkers, cleaved with Not I restriction enzyme, and cloned into pADNS at the Not I site situated between the alcohol dehydrogenase promoter and termination sequences. The use of the rare cutting Not I obviated the need for restriction site methylases commonly used in cDNA cloning.

Approximately 1.5 × 10<sup>5</sup> independent cDNA inserts were contained in the library, with an average insert size of 1.5 kb. DNA prepared from the cDNA expression library was used to transform the RAS2<sup>val19</sup> yeast strain TK161-R2V. The 50,000 Leu<sup>+</sup> transformants obtained were subsequently tested for heat shock sensitivity. Only one transformant displayed heat shock resistance which was conditional upon retention of the expression plasmid. A plasmid, pADPD, was isolated from this transformant and the 2.17-kb Not I insert was analyzed by restriction site mapping (Fig. 1) and nucleotide sequencing (24, 25) (Fig. 2).

A large open reading frame of 562 codons was found. The first ATG, however, appears at codon 46 and a protein that initiates at this codon would have a predicted molecular mass of ~60 KDa. A second shorter open reading frame, separated from the first by three stop codons but in the same frame as the principal coding region, contains 116 codons. The nucleotide sequence of the coding strand ends with a stretch of poly(A). A search for similar sequences was performed and the D. melanogaster dune gene was found. The two genes would encode proteins with an 80% amino acid identity, without the introduction of gaps, over a 252-amino acid region located in the center of the rat DPD cDNA. The dune gene has been shown to encode a high-affinity cAMP phosphodiesterase (30–32).

To demonstrate that the sequences upstream and downstream of the large sequence identity region were in fact contiguous with that region in the mRNA rather than artifacts of our method for cDNA cloning, we compared the structure of our cloned cDNA with DPD cDNAs contained in an independently prepared first-strand cDNA population obtained by reverse transcribing total rat brain poly(A)<sup>+</sup> RNA with an oligo(dT) primer. Oligonucleotide primers complementary to sequences located within the identity region, and to sequences near the 5' or 3' ends of the coding strand, were made. Using either the cloned DNA or the total first-strand cDNA material as template, PCRs were carried out using four different primer sets and the reaction products were analyzed by polyacrylamide gel electrophoresis (Fig. 3). In each case, a fragment of the predicted length was obtained by using either of the template DNAs. The band assignments were confirmed by cleavage with restriction endonucleases having recognition sites within the amplified DNA product. Again, in each case, the primary PCR product obtained using either source of template yielded cleavage products of the predicted sizes. Some submolar background bands do appear in the PCR products but these were unaffected by the restriction digests. The results indicate that the sequence arrangement in the cloned cDNA faithfully reflects the structure of the rat mRNA.

**Expression and Characterization of the DPD Gene Product.** *S. cerevisiae* encodes two cAMP phosphodiesterase genes, *PDE1* and *PDE2* (6, 9). The strain 10DAB carries disruptions of both of these genes. The resulting cAMP phosphodiesterase deficiency leads to elevated intracellular cAMP.
levels and a heat shock-sensitivity phenotype similar to that of strains harboring the RAS*vs11 allele (9). 10DB cells were transformed with the DPD expression plasmid pADPD, were assayed for heat shock sensitivity, and were indeed rendered resistant to heat shock (Fig. 4).

To analyze the biochemical properties of the DPD gene product, crude cell extracts were prepared from 1-liter cultures of 10DB that had been transformed with either pADNS or pADPD. Phosphodiesterase activity assays were performed using cAMP as substrate. Control extracts

FIG. 2. The nucleotide sequence of the DPD cDNA. The top line shows the nucleotide sequence. Below is the predicted amino acid sequence of the open reading frame. Termination codons (indicated with a dot) are located at positions 563, 570, 575, and 692. The bottom row shows the amino acid sequence of the Drosophila duncen gene (28, 38). Identical amino acids in DPD and dance are indicated with triple vertical lines. The area of greatest sequence identity is boxed. Numbers on the left indicate nucleotide and amino acid positions.
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(10DAB with pADNS) showed no cAMP phosphodiesterase activity. Results with the controls were unchanged when performed at 0°C or in the absence of Mg2+ and were comparable to results obtained when no extract was added. These results indicate that there is indeed no detectable background phosphodiesterase activity in this strain.

In contrast, considerable cAMP phosphodiesterase activity was seen in the 10DAB strain transformed with pADPD. The rate of cAMP hydrolysis in cells containing PDH was measured as a function of cAMP concentration (Fig. 5). The deduced \( K_m \) for cAMP is 3.5 \( \mu M \) and the calculated \( V_{max} \) is 1.1 nmol·mg\(^{-1}·\)min\(^{-1}\).

The assay conditions were varied to ascertain the cation preferences of the enzyme and to determine the ability of calcium and calmodulin to stimulate its activity. In these assays, Mn2+ can be used as well as Mg2+, and either cation

![Fig. 3](image.png)

**Fig. 3.** Analysis of DPD cDNA structure using PCR. Reactions were carried out as described and the reaction products (either untreated or cleaved with a restriction endonuclease) were analyzed on a polyacrylamide gel stained with ethidium bromide. The diagram at the bottom of the figure illustrates the DPD cDNA and the positions of primer oligonucleotides (A, B, C, D, E, and F) used. The locations of the restriction sites for Nsi I and Stu I are also shown, as is the position of the termination codons (Ter). Each of the four panels is labeled to indicate the primers used for PCR. Lanes: 1, PCR product using the cloned DNA as template; 2, PCR product resulting from the single-stranded cDNA template made from total rat brain poly(A)+ RNA; 3 and 4, PCR products from the cloned DNA template or cDNA template, respectively, which have been cleaved with Nsi I (AC and BC) or Stu I (DE and DF). Restriction fragment lengths are indicated on the left. These lengths have been calculated from the known sequence and are in agreement with the observed mobility of standard DNA fragments run on the same gel.

![Fig. 4](image.png)

**Fig. 4.** Heat shock phenotype. Two heat shock-sensitive yeast strains, TK161-R2V (RAS2*319) and 10DAB (pde1, pde2*), were transformed with either the pADNS cloning vector alone (control), or with the pADPD plasmid expressing the rat brain phosphodiesterase (DPD). Yeast patches were grown on synthetic medium plates for 1 day and then replica plated to fresh plates at 30°C (Left) or to preheated plates and incubated at 55°C for 10 min before returning to 30°C (Right). Recovery time at 30°C was 36 hr.

**Fig. 5.** DPD enzyme kinetics. Phosphodiesterase assays were performed on cell extracts as described with a final Mg2+ concentration of 10 mM. The cAMP concentration was varied from 0.2 to 10 \( \mu M \). Two independent determinations were made and background measured in vector-only extracts was subtracted. Error bars are based on SD.

in 1 mM final concentration was sufficient. Calcium/calmodulin was unable to stimulate the measured phosphodiesterase activity in the extract (data not shown). A parallel assay in which beef heart phosphodiesterase (Boehringer Mannheim) was used yielded a 6.5-fold stimulation with the addition of calcium/calmodulin (data not shown). Finally, no cGMP phosphodiesterase activity was detected in our assays. Beef heart phosphodiesterase was again used as a positive control. In addition, cGMP present in amounts 100-fold over substrate concentrations was unable to inhibit cAMP phosphodiesterase activity.

**DISCUSSION**

Previous workers have cloned a mammalian gene in yeast by using a biological screen (33). In that case, a homolog to the cdc2 gene of Schizosaccharomyces pombe was cloned by screening a cDNA library for complementation of cdc2 mutants. In that library, the cDNAs were inserted proximal to the simian virus 40 early large tumor antigen promoter. In our work, we have used a library with mammalian cDNAs inserted into a yeast expression vector, proximal to a strong yeast promoter. In addition, we have used Not I linkers for cDNA cloning, which allows the convenient subcloning of an entire insert library from one vector to another. We feel that this will be a generally useful approach for cloning genes from higher eukaryotes when functional screens are possible in yeast. This system is particularly useful for the cloning of other cAMP phosphodiesterases from mammals. The availability of yeast strains totally lacking endogenous cAMP phosphodiesterase activity will also facilitate the biochemical characterization of these new phosphodiesterases.

The mammalian DPD cDNA can encode a protein with a high degree of amino acid sequence identity (80%) with the predicted D. melanogaster ducne gene product over an extended region. The ducne gene has been shown to encode a high-affinity cAMP phosphodiesterase required for normal learning and memory in flies (30–32). Compared to the striking level of sequence identity between DPD and ducne, the sequence conservation among other known cAMP phosphodiesterases is scant (34). Therefore, the DPD–ducne homology in the conserved region represents more than a constraint on sequences required for cAMP binding and hydrolysis and suggests a conservation of interactions with other components.
Biochemical characterization of the DPD cDNA product expressed in yeast indicates that it is a high-affinity cAMP-specific phosphodiesterase, as is Dunce (31, 32). In addition, DPD activity, as measured in our assays, is not stimulated by the presence of calcium/calmodulin. This property is shared with Dunce and is distinct from some other phosphodiesterases (for a review, see ref. 35). The two proteins, DPD and Dunce, thus appear to have similar biochemical characteristics. However, it should also be noted that DPD encodes a protein product that shows much less significant homology (35%) to Dunce beyond the previously described highly conserved region. These nonconserved sequences could result in an altered or refined function for this mammalian Dunce homolog.

Since the predicted rat DPD protein diverges from the Drosophila Dunce gene product, and since we have merely a single cDNA isolate, we were concerned that the structure of our cDNA might not reflect the structure of the DPD mRNA. We have described here the use of PCRs to compare the structure of our DPD cDNA with the DPD mRNA. This study indicates a complete concordance in structure. Our method should also be applicable to the detection and analysis of alternate mRNA splicing (see below).

Our DPD sequence encodes a methionine codon at position 46 and the established reading frame remains open through to position 563, resulting in a protein with a predicted molecular mass of 60 kDa. The same reading frame, however, is open beyond the 5′ end of the coding strand (Fig. 2). At present, we cannot say whether the methionine codon at position 46 is the initiating codon for the DPD protein. The coding sequence is interrupted by three closely spaced terminator codons. However, the established reading frame then remains open for an additional 116 codons, followed by more terminator codons, a polyadenylation consensus signal, and a poly(A) stretch. This 3′ open reading frame could be incorporated into another Dunce-like phosphodiesterase through alternate splicing. To examine this, we utilized the PCR method by using oligonucleotides from the conserved region and from the downstream open reading frame [oligo(D) and -F, respectively, in Fig. 3]. Our PCR method reveals no evidence of a DPD mRNA in adult rat brain that utilizes both the highly conserved domain and the open reading frame 3′ to the stop codons. It should be noted, however, that a complex transcription pattern involving alternately spliced messages has been described for the D. melanogaster Dunce locus (36, 37), and this may also be a feature of the mammalian homolog.

Davis et al. (38) have recently isolated a mammalian Dunce homolog from a rat brain cDNA library by standard nucleic acid hybridization techniques. The gene they describe is indeed similar to, though distinct from, the DPD cDNA described here. Within the highly conserved region, as defined in this work, the predicted amino acid sequences of the two rat genes are 93% identical. This homology falls off dramatically, however, in the flanking regions, which show amino acid identities of 60% (upstream) and 30% (downstream) and require the use of sequence gaps for optimum alignment. These differences should be sufficient to distinguish the two related messages in situ hybridizations and to permit the study of their distribution throughout the adult rat brain and during development.

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