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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Communicated by James D. Watson, February 13, 1989

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 $RAS2^{val19}$, a mutant form of the RAS2 gene analogous to an oncogenic mutant of the human HRAS gene. One cDNA, DPD, was cloned and its genetic and biochemical properties were characterized. A DPD product would share 80% amino acid sequence identity with the Drosophila melanogaster dunce-encoded protein over an extended region. We have shown that the DPD protein is a high-affinity cAMPspecific phosphodiesterase.

The yeast Saccharomyces cerevisiae encodes two genes, RASI and RAS2, that have structural and functional homology with mammalian RAS oncogenes (1–4). When an activated form of the RAS2 gene (RAS2^{val19}) is present, yeast cells fail to synthesize glycogen, 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 adenylyl 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 $RAS2^{val19}$ 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, *DPD* (dunce-like phosphodiesterase), encodes a high-affinity cAMP phosphodiesterase that is highly homologous to the cAMP phosphodiesterase encoded by the *dunce* locus of *Drosophila melanogaster*. *D. melanogaster* with mutations in *dunce* 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 SCS1 (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 ras1::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 ras1::HIS3) was subsequently transformed with the 5.4-kilobase-pair (kbp) Xba I pde1::ADE8 fragment of pYT19-

DAB 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 PDE1 sequences between the Sma 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 $2-\mu m$ 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 (ADH1) promoter (22, 23) and a 0.6-kbp HindIII/BamHI fragment containing the ADH1 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'-GG-CCAAAAAGGCCGCGGCCGCA and 5'-TCGACCGGTTT-TTCCGGCGCCGGCGTTCGA. The plasmid pADPD 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 *dunce* 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'-AAGCG-GCCGC and 5'-GCGGCCGCTT. The cDNAs were cleaved with *Not* I and cloned into the *Not* I site of pADNS by standard procedures.

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

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Abbreviation: PCR, polymerase chain reaction.

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[†]The 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 μ g of total first strand cDNA), 25 pmol of oligonucleotide primers, 200 μ M 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'-CACCCTGCTGACAAACCT⁴⁴; B, 5'-ATGG-AGACGCTGGAGGAA¹⁵³; C, 5'-ATACGCCACATCAGA-ATG⁶⁷⁶; D, 5'-TACCAGAGTATGATTCCC¹⁴⁴⁹; E, 5'-GTG-TCGATCAGAGACTTG¹⁶⁶⁸; F, 5'-GCACACAGGTTGGC-AGAC²⁰⁴⁸. 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 μ l of 1 M phenylmethylsulfonyl fluoride, and were disrupted with a French press. The extracts were centrifuged at $1600 \times g$ for 10 min and the supernatants were spun at $18,000 \times g$ 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 μ g of protein per ml), 5'-nucleotidase (20 ng/ml; Sigma), and Mg²⁺ (10 mM) (unless otherwise stated) and the indicated cyclic nucleotide concentrations. Assays for cGMP hydrolysis used 1.5 µM cGMP. Inhibition studies used 5 μ M cAMP in the presence of various amounts of cGMP up to 500 μ M. [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 $RAS2^{val19}$ 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 $RAS2^{val19}$ expressing strain TK161-R2V (6, 9). We have now used the same selection to isolate mammalian genes that can function in yeast to render $RAS2^{val19}$ 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 1.5×10^5 independent cDNA inserts were contained in the library, with an average insert size of 1.5 kbp. DNA prepared from the cDNA expression library was used to transform the *RAS2*^{val19} yeast strain TK161-R2V. The 50,000 Leu⁺ 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



IOObp

FIG. 1. Restriction maps of expression vector and isolated insert. (A) The expression vector pADNS is described in detail in *Materials* and Methods. It contains selectable markers for use in yeast (LEU2), and bacteria (AMP^R), as well as yeast and bacterial origins of replication. The yeast alcohol dehydrogenase sequences are shown with the Not I cloning site located between them. (B) The DPD cDNA insert is shown as a 2.17-kbp Not I fragment. N, Not I; Ns, Nsi I; D, Dra I; P, Pvu II; Sa, Sac I; K, Kpn I; S, Stu I; X, Xba I. The cDNA is presented with the coding strand oriented 5' (left) to 3' (right).

poly(A). A search for similar sequences was performed and the *D. melanogaster dunce* 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 *dunce* 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)⁺ 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

1 1 1	AGC Ser	TTG Leu Met	CGA Arg Phe	ATC Ile Gln	GTA Val His	AGA Arg Gln	AAC Asn Thr	AAT Asn 1 Asn 1	TTC Phe Pro	ACC Thr Gly	CTG Leu Gly	CTG Leu Pro	ACA Thr Thr	AAC Asn Asn	CTT Leu	CAC His	GGA Gly	GCA Ala	CCG Pro	AAC Asn Arg	AAG Lys Arg	AGG Arg Arg	TCG Ser Pro	CCA Pro Arg	GCG Ala Asp	GCT Ala Gln	AGT Ser Glu	CAG Gln Ile	GCT Ala His	CCA Pro Gln	GTC Val Glu	ACC Thr Pro	AGA Arg Arg	GTC Val Tyr	AGC Ser Pro	CTG Leu Lys
109 37	C AA Gln	GAA Glu	G AA Glu	TCA Ser	TAT Tyr	C A G Gln	AAA Lys	CTA Leu	GCA Ala	ATG Met	G A G Glu	ACG Thr	CTG Leu	GAG Glu	GAA Glu	CTA Leu	GAC Asp	TGG Trp	TGC Cys	CTA Leu	GAC Asp	C A G Gln	CTA Leu	GAG Glu	ACC Thr	ATC Ile	C AG Gln	ACC Thr	TAC Tyr	CGC Arg	TCT Ser	GTC Val	AGC Ser	GAG Glu	ATG Met	GCT Ala
31	Ala	Arg	Gly	His	Thr	Pro	Ala	Trp	Pro	Pro	Thr	Gln	Ser	Arg	Ser	Trp	Thr	Gly	Ala	Ser	Thr	Ser	Trp	Arg	Pro	Ser	Arg	Pro	Ile	Ala	Ala	Ser	Pro	Thr	Trp	Arg
217 73 67	TCA Ser Arg	AAC Asn Leu	AMG Lys Ser	TTC Phe Cys	AAA Lys Lys	AGG Arg Arg	ATG Met Met	CTG Leu Leu	AAC Asn Asn	CGG Arg Lys	GAG Glu Glu	CTG Leu Leu	ACA Thr Ser	CAC His His	CTC Leu Phe	TCA Ser III Ser	GAG Glu Glu	ATG Met Ser	AGC Ser []] Ser	AGA Arg Arg	TCA Ser Ser	GGG Gly []] Gly	AAC Asn Asn	C AA Gln Gln	GTG Val Ile	TCT Ser Ser	GAA Glu Glu	TAC Tyr Tyr	ATT Ile Ile	TCG Ser Cys	AAC Asn Ser	ACG Thr Thr	TTC Phe Phe	TTA Leu iii Leu	GAC Авр Авр	AAG Lys Lys
325 109 103	CAG Gln Gln	AAC Asn Gln	GAT Asp Glu	GTG Val Phe	GAA Glu Asp	ATC Ile Leu	CCA Pro Pro	TCT Ser Ser	Leu	A rg	Val	Glu	Asp	As n	CCC Pro Pro	ACC Thr Glu	CAG Gln Leu	AAG Lys Val	GAC Asp Ala	AGG Arg Ala	GAG Glu Asn	AAG Lys Ala	AAG Lys Ala	AAG Lys Ala	AAG Lys Gly	CAG Gln Gln	CAG Gln Gln	Ser	A la	Gly	Gln	Tyr	Ala	A rg	Ser	Arg
388 130 145	Ser	Pro	Arg	Gly	Pro	CTC Leu Pro	ATG Met Met	ACC Thr Ser	CAG Gln Gln	ATA Ile Ile	AGT Ser Ser	GGA Gly Gly	GTG Val Val	AAG Lys Lys	A rg	AAA Lys Pro	CTG Leu Leu	ATG Met Ser	CAC His His	AGC Ser Thr	TCA Ser Asn	AGC Ser III Ser	CTG Leu Phe	AAC Asn Thr	AAC Asn Gly	ACA Thr Glu	AGC Ser Arg	ATC Ile Leu	TCA Ser Pro	CGC Arg Thr	TTT Phe Phe	GGA Gly Gly	GTC Val Val	AAC Asn Glu	ACG Thr Thr	G AA Glu Pro
478 160 181	AAT Asn Arg	GAG Glu Glu	GAT Asp Asn	CAT His Glu	CTA Leu Leu	GCC Ala Gly	AAG Lys Thr	GAG Glu Leu	CTG Leu Leu	GAA Glu Gly	GAC Asp Glu	CTG Leu Leu	AAC Asn Asp	AAA Lys Thr	TGG Trp Trp	GGC Gly Gly	CTT Leu Ile	AAC Asn Gln	ATC Ile III Ile	TTC Phe Phe	AAC Asn Ser	GTG Val Ile	GCT Ala Gly	GGG Gly Glu	TAC Tyr Phe	TCC Ser III Ser	CAT His Val	AAT Asn Asn	CGG Arg Arg	CCC Pro 111 Pro	CTC Leu III Leu	ACA Thr Thr	TGC Cys Cys	ATC Ile Val	ATG Met Ala	TAC Tyr Tyr
586 196 217	GCC Ala Thr	ATT Ile Ill Ile	TTC Phe !!! Phe	CAG Gln Gln	GAA Glu Ser	AGA Arg Arg	GAC Asp Glu	CTT Leu Leu	CTA Leu Leu	AAG Lys Thr	ACG Thr Ser	TTT Phe Leu	AAA Lys Met	ATC Ile Ile	TCC Ser Pro	TCC Ser Pro	GAC Asp Lys	ACC Thr Thr	TTC Phe 111 Phe	GTA Val Leu	ACC Thr Asn	TAC Tyr Phe	ATG Met Net	ATG Met Ser	ACT Thr Thr	TTA Leu Leu	GAA Glu Glu	GAC Asp Asp	CAT His His	TAC Tyr Tyr	CAT His Val	TCT Ser Lys	GAT Asp Asp	GTG Val Asn	GCG Ala Pro	TAT Tyr Phe
694 232 253	CAC His His	AAC Asn Asn	AGC Ser 111 Ser	CTG Leu Leu	CAC His His	GCT Ala Ala	GCT Ala Ala	GAC Asp Asp	GTG Val Val	GCC Ala Thr	CAG Gln Gln	TCA Ser Ser	ACG Thr Thr	CAC His Asn	GTT Val Val	CTC Leu III Leu	CTC Leu III Leu	TCT Ser Asn	ACG Thr Thr	CCA Pro Pro	GCA Ala III Ala	CTG Leu Leu	GAT Asp Glu	GCT Ala Gly	GTC Val III Val	TTC Phe III Phe	ACA Thr Thr	GAC Asp Pro	CTG Leu Leu	GAA Glu Glu	ATC Ile Val	CTG Leu Gly	GCT Ala Gly	GCC Ala III Ala	ATT Ile Leu	TTT Phe Phe
802 268 289	GCA Ala Ala	GCT Ala III Ala	GCC Ala Cys	ATC Ile Ile	CAT His His	GAT Asp Asp	GTT Val Val	GAT Asp Asp	CAT His His	CCT Pro III Pro	GGA Gly Gly	GTC Val Leu	TCC Ser Thr	AAT Asn Asn	CAG Gln Gln	TTT Phe Phe	CTC Leu Leu	ATC Ile Val	AAT Asn Asn	ACA Thr Ser	AAT Asn Ser	TCC Ser Ser	GAA Glu Glu	CTT Leu Leu	GCT Ala Ala	TTG Leu Leu	ATG Met Met	TAT Tyr Tyr	AAT Asn Asn	GAC Asp Asp	GAA Glu Glu	TCT Ser Ser	GTG Val Val	CTG Leu Leu	GAA Glu Glu	AAC Asn Asn
910 304 325	CAT His His	CAC His []] His	CTC Leu Leu	GCT Ala Ala	GTG Val Val	GGA Gly Ala	TTC Phe Phe	AAG Lys Lys	CTC Leu Leu	CTT Leu Leu	C AA Gln Gln	GAG Glu Asn	Ġ AA Glu Gln	CAT His Gly	TGC Cys Cys	GAC Asp Asp	ATC Ile Ill Ile	TTT Phe Phe	CAG Gln Cys	AAT Asn Asn	CTT Leu Met	ACC Thr Gln	Lys Lys Lys	Lys Lys Lii Lys	Gln Gln Gln Gln	CGC Arg Arg	CAG Gln Gln	ACA Thr Thr	CTC Leu Leu	AGG Arg Arg	Lys Lys Lys	ATG Met Met	GTG Val Val	ATT Ile Ile	GAC Asp Asp	ATG Met Ile
1018 340 361	GTG Val iii Val	TTA Leu Leu	GCA Ala Ser	ACT Thr Thr	GAT Asp Asp	ATG Met Met	TCC Ser Ser	AAG Lys Lys	CAC His His	ATG Met Met	AGC Ser Ser	CTC Leu Leu	CTG Leu III Leu	GCT Ala Ala	GAC Asp Asp	CTT Leu Leu	AAA Lys Lys	ACG Thr Thr	ATG Met Met	GTA Val Val	GAA Glu Glu	ACC Thr Thr	Lys Lys Lys	Lys Lys III Lys	GTG Val Val	ACG Thr Ala	AGC Ser Gly	TCC Ser Ser	GGT Gly Gly	GTT Val III Val	CTC Leu Leu	CTC Leu Leu	CTG Leu Leu	GAC Asp Asp	AAC Asn Asn	TAT Tyr Tyr
1126 376 397	ACT Thr Thr	GAC Asp Asp	CGG Arg Arg	ATA	CAG Gln Gln	GTT Val III Val	CTI Leu III Leu	CGC Arg	AAC Asn Asn	ATG Met Leu	GTA Val Val	CAT His His	TGT Cys Cys	GCA Ala Ala	GAC Asp Asp	CTG Leu Leu	AGC Ser Ser	AAC Asn Asn	CCT Pro Pro	ACC Thr Thr	AAG Lys Lys	TCC Ser Pro	E TTO Lev III	G GAG G Glu G Pro	E TTG Leu Leu	TAT Tyr Tyr	CGG Arg Lys	CAA Gln Arg	TGG Trp Trp	ACT Thr Val	GAT Asp Ala	CGC Arg	ATC Ile Leu	: ATG > Met 1 Met	GAG Glu III Glu	GAG Glu III Glu
1234 412 433	TTT Phe Phe	Phe Phe	CAN Glr Leu	CAG Gln Gln Gln	603 619 111 619	GAC Asp III Asp	Lya Lya Lya	GAA Glu Glu	CGG Arg Arg	GAG Glu Glu	AGG Arg Ser	GGA Gly Gly	ATG Met Met	GAG Glu Asp	ATT 110 111 110	AGC Ser Ser	CCA Pro 111 Pro	ATG Met	TGT Cys Cys	GAT Asp Asp	AAA Lys Arg	CAC His His	C ACJ 5 Thi 6 Asi	A GCT F Ala III h Ala	f TCT a Ser a Thi	GTG Val	GAA Glu Glu	AAG Lys Lys	Ser Ser Ser Ser	CAG Glr III Glr	GTI Val IIII Val	GGT Gly HII Gly	Phe Phe Phe	: ATT : Ile : !!! : Ile	GAC Asp Asp	TAC Tyr 111 Tyr
1342 448 469	ATT Ile III	GTC Val	CA1 His His	CCJ Pro	TTG Leu III	: TGG : Trp : : Trp	GAG	ACC Thr III	TGG Trp Trp	GCA Ala Ala	GAC Asp Ser	CTG Leu Leu	GTT Val III Val	CAG Gln His	CCT Pro Pro	GAT Asp Asp	GCT Als III	CAA Gln Gln	GAC Asp Asp	ATT 110 111 111	TTG Leu Leu	GAC Asp II Asp	CACI Thi III Thi	CTJ r Let I II r Let	A GAJ J Glu J Glu J Glu	A GAT Asp Glu	AAC Asn Asn	AGG Arg Arg	i AAC J Ast J Ast	TGC Trj	TAC Type Type Type Type	Glr Glr Glr	AG1 Sei III Sei	: ATG : Met : Met	; ATT : Ile : Ile : Ile	CCC Pro III Pro
1450 484 505	CAG Glr	AGC Sei	Pro	Ser	Pro	Pro	CTG Let	GAC Asp Ser	Glu Glu	AGG Arg Val	AGC Ser Asp	AGG Arg Glu	GAC Asp Asn	TGC Cys Pro	CAN Gln Gln	GGC Gly Glu	CTI Leu Asp	ATG Met	GAG Glu Ile	AAG Lys Arg	; TTT Phe Fhe	CAG Glr Glr	3 1 1 Va:	1 T hi	r Lev	ı Glu	a Glu	Ser	. Yel	o Gla	n Glu	TTC Phe	GAN Glu Leu	L CTG 1 Leu 1 Ali	; ACC 1 Thi Glu	: CTT : Leu J Leu
1531 511 539	GAU Glu Glu	Glu Glu Glu Glu	Glu Glu Gly	GA1 1 Asp 7 Asp	Ser Glu	GAL Glu Sei	603 1 613 1 1 1 1 1 1 1 613	CCG Pro Gly	Glu Glu Glu	ANG Lys Thr	GAG Glu Thr	GGM Gly Thr	GAN Glu Thr	GGC Gly Gly	Pro Thr	AMC Asn Thr	TAT Tyr Gly	TTC Phe Thr	Ser Ser	AGC Ser Als	Thr	Lya Sei	G ACI B Thi F Ala	A CT: r Lei a Lei	T TG: L Cyr	f GTG 9 Val 9 Ala	ATC 110 Gly	GAT Asp Gly	r ccr p Pro	GIN GIN GIN	5 AA 1 Asi 7 G1	AGG Arg Gly	GAN JASI JG1	r TCT p Ser y Glj	r CTO r Leu y Glj	; GAA 1 Glu 7 Met
1639 547 575	GAG Glu Ala	Der Pro	GAG Asj Arg	Thi	. GAG Asp : Gly	Gly	r GCG Ali 7 Cyr	C ACM	Glu Glu Asr	GAC Asp Glr	AAG Lys Pro	Glr	CTG Leu His	Gly	GAC Asp Gly	ACA Thr Net	71	Ser	f CCC F Pro	TC1 Ser	GTG Val	: 160 . Trj	S AG	G TGJ g .	A ACI Thi	A TTO	TAI Tyr	Pro	TGJ	A CG	A GCI J Ali	t TGC	C CAA	; CTC a Leu	3 AG1 1 Sei	f GGT f Gly
1747 583	AGO Arg	GCC 7 Ale	CAC	CTJ	CCI Pro	6 G10	CCI Pro	A AGG	CC1	GCJ	CAN Gln	AAC Asr	Lys	GGC	CAC Bis	CTG	GCT	TTG Leu	CAG	TTI Leu	CTI Lau	GAA	G TT u Ph	T GG	A GC	C AG	a Arg	CAJ Glr	A GGG	C CG	T GA	A GCI	1 77 1 79	T AGA	C AG	f TCC r Ser
1855 619	GTG Val	CTG Let	CC Pro	TGC Cyr	CTI Leu	GCC Ale	: 660 61)	GAG Glu	CTI Let	660 613	GAG Glu	ACC Tha	CGC Arg	AGC Sei	TG1 Cys	AG1 Sei	AGJ	AGC J Sei	C.M.	TTC Phe	CCI Pro	GCI Ala	A CA	G CT n Ler	а аа 11 ав:	T GGG n Gl	C TTG Y Leu	i AAJ	A ACI	A GA	G GA	AG	J Ly	G CTO	G AGI	A GAT 3 Asp
1963 655	TGC Cyr	TC: Sei	GCI Ali	A ATA	. 661 6 613	r GT1 7 Val	GAC Glu	GCG Gly	CTC Let	TCC Sel	CGA Arg	CAG Glr	GTG Val	ACT	Glu	CTC Let	AC1	: 330 : 381	ANC	TTC	Ile	: TA 5 Ty:	T AA T Ly	A TC S Sei	T CA F Bi	C CC	A 700 Sei	TG1	T TG: Cy	I CI	G CC.	A AC	C TG	T GT S Va	S CC	f TTT o Phe
2071 691	TTC Let	; 7 .)		GTT	TCG	GTCI	TTG	LAATG	CCTG	TTGJ	ATAT	CTAG	AGTI	TAGI	ACCI	CCTI	CTAC			TTGJ	GICI	TTC	TGGG.	****	,,,,,		N.									

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 dunce* gene (28, 38). Identical amino acids in *DPD* and *dunce* are indicated with triple vertical lines. The area of greatest sequence identity is boxed. Numbers on the left indicate nucleotide and amino acid positions.

levels and a heat shock-sensitivity phenotype similar to that of strains harboring the $RAS2^{val19}$ allele (9). 10DAB 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).

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

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Proc. Natl. Acad. Sci. USA 86 (1989)



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.

(10DAB with pADNS) showed no cAMP phosphodiesterase activity. Results with the controls were unchanged when performed at 0°C or in the absence of Mg^{2+} 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 *DPD* was measured as a function of cAMP concentration (Fig. 5). The deduced K_m for cAMP is 3.5 μ 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, Mn^{2+} can be used as well as Mg^{2+} , and either cation



FIG. 4. Heat shock phenotype. Two heat shock-sensitive yeast strains, TK161-R2V ($RAS2^{val19}$) 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 Mg^{2+} concentration of 10 mM. The cAMP concentration was varied from 0.2 to 10 μ 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 dunce* gene product over an extended region. The *dunce* 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 *dunce*, the sequence conservation among other known cAMP phosphodiesterases is scant (34). Therefore, the *DPD*-*dunce* 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 cAMPspecific 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 product 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 polyadenylylation 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 *in situ* hybridizations and to permit the study of their distribution throughout the adult rat brain and during development.

We thank Ronald L. Davis for sharing his results prior to publication. We also thank Ilse Wieland and Kenneth Ferguson for technical advice. We also thank Patricia Bird for her help in preparation of this manuscript. This work was supported by grants from the National Institutes of Health, the Pfizer Biomedical Research Award, the American Cancer Society, and the American Business Foundation for Cancer Research. J.C. is a Schering-Plough Foundation Fellow of the Life Sciences Research Foundation, T.M. is supported by the Damon Runyon–Walter Winchell Cancer Fund, and M.W. is an American Cancer Society Professor.

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