Isolation of a cDNA Encoding a Portion of the Cyclic Nucleotide Phosphodiesterase of *Dictyostelium discoideum*

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The cyclic nucleotide phosphodiesterase (phosphodiesterase) of *Dictyostelium discoideum* is one of a group of developmentally regulated proteins which enable cells to aggregate by chemotaxis during the early stages of development. We report the identification and DNA sequence of a cDNA clone encoding the amino-terminal region of the phosphodiesterase. The clone, pPD-3, was selected from a cDNA library created by priming first strand synthesis using a set of oligonucleotides with sequences predicted from the amino-terminal amino acid sequence of purified phosphodiesterase. The DNA sequence of pPD-3 encodes perfectly the available phosphodiesterase amino acid sequence, and pPD-3 selects an mRNA which can be translated into material recognized by phosphodiesterase antisera. The nucleotide sequence of pPD-3 indicates there are 49 amino acids, which contain a segment possessing the characteristics of a signal peptide, that separate the amino-terminal residue identified in the purified protein from the methionine codon at which translation originates. DNA blot analysis demonstrates that the phosphodiesterase gene exists as a single copy in the nuclear genome. Analysis of RNA indicates that the phosphodiesterase transcript is 2.1 kb long, which is approximately 0.8 kb more than the minimum required to encode this protein.

INTRODUCTION

The cyclic nucleotide phosphodiesterase (EC 3.1.4.17) (phosphodiesterase) of *Dictyostelium discoideum* is one of a group of proteins elaborated during development that function coordinately to allow amoebae to detect and move toward cells which secrete cyclic AMP (cAMP). cAMP plays multiple roles during the development of this organism, functioning as a chemoattractant to draw cells into aggregates (Devreotes, 1982) and as a regulator of gene expression (Mehdy *et al.*, 1983). To maintain the responsiveness of cells to cAMP, concentrations of the nucleotide must be kept below levels which saturate cAMP receptors. Phosphodiesterase fulfils this function by hydrolysing the cAMP produced by developing cells. The requirement for cAMP hydrolysis is demonstrated by mutants deficient in the production of phosphodiesterase which are unable to complete the early stages of cell aggregation unless provided with an exogenous source of this enzyme (Barra *et al.*, 1980).

Phosphodiesterase has been purified to homogeneity, and can be resolved into two components of 48 and 50 kDa which probably differ only in the extent of their glycosylation (Orlow *et al.*, 1981). The enzyme may be secreted or associated with the membrane. The membrane-bound and extracellular enzymes are similar by several criteria including M_r , peptide map, kinetic properties, substrate and inhibitor specificities, and antigenic properties (Shapiro *et al.*, 1983).

The phosphodiesterase of *D. discoideum* offers an opportunity to study mechanisms which regulate the activity of developmentally expressed genes. Phosphodiesterase transcripts are found at low levels during vegetative growth and increase in abundance during the early stages

of development (Mullens *et al.*, 1984). Transcription of the phosphodiesterase gene is stimulated by cAMP, as exposure of starved cells to the nucleotide induces the synthesis of phosphodiesterase messenger RNA, and results in the coordinate appearance of phosphodiesterase enzyme activity (Mullens *et al.*, 1984). The enzyme may be directed preferentially to the membrane or secreted from the cell depending on whether the cells are exposed to pulses or steady state concentrations of cAMP (Yeh *et al.*, 1978). The activity of the secreted, but not the membrane-bound enzyme is also controlled by interaction with a specific inhibitory glycoprotein which is elaborated during development (Kessin *et al.*, 1979; Franke & Kessin, 1981).

We have begun work to isolate the phosphodiesterase gene to help understand the mechanisms that control expression of the gene and direct the localization of the enzyme. Here we report the isolation and nucleotide sequence of a cDNA encoding the amino-terminal portion of the *D. discoideum* phosphodiesterase.

METHODS

Amino acid sequencing. The amino-terminal amino acid sequence of phosphodiesterase purified according to Orlow *et al.* (1981) was determined using an Applied Biosystems model 470A sequenator. Phenylhydantoin amino acid derivatives were analysed using a Hewlett-Packard model 1090 HPLC.

Preparation of DNA and RNA. All nucleic acids were isolated from *D. discoideum* strain AX3 grown in HL/5 medium (Franke & Kessin, 1977). DNA was extracted from isolated nuclei by the method of Welker *et al.* (1985). RNA was obtained by the method of Alton & Lodish (1977) from cells harvested after shaking for 3 h in 17 mM-KH₂PO₄/NA₂HPO₄ buffer (pH 6.0) containing 0.5 mM-CaCl₂ and 1 mM-cAMP. RNA enriched for poly(A)⁺ sequences was obtained by chromatography on oligo(dT)-cellulose (Collaborative Research Laboratories).

Construction and screening of the cDNA library. Mixed oligonucleotides prepared by P-L Biochemical Co. were labelled by 5' addition of ³²P using [γ-³²P]ATP [New England Nuclear; 3000 Ci mmol⁻¹ (111 TBq mmol⁻¹)] and T₄ polynucleotide kinase (Bethesda Research Laboratories) (Maxam & Gilbert, 1977). Reverse transcription was done in a volume of 100 µl containing 25 µg poly(A)⁺ RNA, 225 pmol mixed 17-base primer and 90 U avian myeloblastosis reverse transcriptase (Life Sciences) using conditions described by Merritt *et al.* (1983). Conditions for second strand synthesis using Klenow fragment (New England Biolabs) and S₁ nuclease (Bethesda Research Laboratories) treatment were as described by Maniatis *et al.* (1982). The cDNA was tailed with dCTP using terminal deoxynucleotidyl transferase (Bethesda Research Laboratories) according to instructions provided by the manufacturer, and annealed with oligo(dG)-tailed *Pst*I-cleaved pBR322 (Bethesda Research Laboratories) before being used to transform *Escherichia coli* strain DH1 (Hanahan, 1983). Colonies were selected on LB agar containing 12.5 µg tetracycline ml⁻¹ and transferred to Colony-Plaque Screen disks (New England Nuclear) according to directions given by the manufacturer. Replicas were prepared and incubated overnight on LB agar containing 170 µg chloramphenicol ml⁻¹ (Hanahan & Meselson, 1983). Following colony lysis, replica disks were prehybridized for 6 h at the hybridization temperature in a solution of 6 × SSC (1 × SSC = 0.15 M-NaCl, 0.015 M citrate (pH 7.0)), 0.1% SDS, 0.1% Ficoll (*M*_w 400 000), 0.1% polyvinylpyrrolidone (*M*_w 40 000) and 0.1% bovine serum albumin. Radiolabelled oligonucleotide probe was added to yield approximately 5 × 10⁵ cpm ml⁻¹ for overnight hybridization either at 43 °C when using the 17-base oligonucleotide, or at 26 °C when using the 14-base oligonucleotide. Excess probe was removed by washing once at 4 °C for 10 min in 6 × SSC, then twice for 1 h at 4 °C in 2 × SSC. Filters were dried and used with intensifying screens to expose Kodak XAR-5 film at -80 °C for 12-48 h.

DNA sequence analysis. Following cleavage of recombinant plasmids with *Pst*I to excise cDNA, DNA was strand-separated by heating to 100 °C in the presence of a 20-fold molar excess of the mixed 17-base oligonucleotide used in cDNA library construction. This mixture was annealed by cooling on ice to 0 °C, and the nucleotide sequence was determined beginning at the 17-base primer using the dideoxy chain termination method with [α-³⁵S]dATP [650 Ci mmol⁻¹ (24 TBq mmol⁻¹); Amersham] as described by Biggin *et al.* (1983).

Preparation of phosphodiesterase-specific probe. ³²P-labelled probe was prepared by primer extension from the 17-base oligonucleotide used in the construction of the cDNA library, using as template a fragment produced by *Taq*I digestion of the purified cDNA insert of pPD-3 which had been excised by digestion with *Pst*I. *Taq*I digestion of pPD-3 cDNA produced a fragment approximately 240 nucleotides long extending from nucleotide -29 shown in Fig. 1 to the 3' flanking *Pst*I site. Conditions for primer extension were those described above for DNA sequence analysis utilizing three unlabelled dNTP's and [α-³²P]dATP [800 Ci mmol⁻¹ (30 TBq mmol⁻¹); New England Nuclear]. This procedure eliminated oligo(dG)-oligo(dC) regions from the probe, and typically produced probe with a specific activity in excess of 1 × 10⁸ c.p.m. µg⁻¹.

Hybridization selection: Recombinant plasmids were bound to nitrocellulose and hybridized with poly(A)+ RNA as described by Fishel *et al.* (1985). Hybridized RNA was eluted, translated *in vitro*, precipitated with anti-phosphodiesterase serum, and analysed according to Mullens *et al.* (1984).

RNA analysis. Poly(A)+ RNA (10 µg) was treated with glyoxal, separated by electrophoresis through a 1% (w/v) agarose gel, and transferred to a Gene Screen Plus (New England Nuclear) membrane according to instructions provided by the manufacturer. The blot was prehybridized for 6 h at 55 °C in a solution of 1 M-NaCl, 1% (w/v) SDS before addition of a heat-denatured mixture of ³²P-labelled probe and sufficient herring sperm DNA to yield a final DNA concentration of 100 µg ml⁻¹. Hybridization was continued at 55 °C for 16 h before washing according to Raymond *et al.* (1984). The dried blot was used with intensifying screens to expose Kodak XAR-5 film at -80 °C for 24 h.

RESULTS

Isolation of phosphodiesterase cDNA

A continuous sequence of amino acids extending from residues 3 to 23 of extracellular phosphodiesterase was determined by automated Edman degradation, and this information was used in the design of two 16-member families of oligonucleotides 14 and 17 bases long. These oligonucleotides were complementary to all possible codon choices in non-overlapping regions of the potential mRNA sequence (Fig. 1). Amino acids 3 to 23 of the purified processed protein correspond to amino acids 52 to 72 of the primary translation product later deduced from the nucleotide sequence of pPD-3. The stretch of amino acids sequenced by Edman degradation is shown in capital letters in Fig. 1. We chose a cloning strategy to enrich for phosphodiesterase cDNA because the phosphodiesterase transcript is rare, comprising at most 0.015% of translatable mRNA present during maximal induction (Mullens *et al.*, 1984), and because the protein sequence available for the design of probes corresponded to the 5' region of the mRNA. This portion of the RNA would not be represented in truncated cDNA molecules primed from the 3' poly(A) tail with oligo d(T).

To create a cDNA library, we used the 17-base oligonucleotide as a primer for first strand cDNA synthesis. The library was screened initially using the 14-base probe, and colonies which hybridized to the probe were purified and rescreened with both the 14-base and 17-base oligonucleotides. Approximately 1 in 200 colonies hybridized to both probes. Analysis of the cDNA inserts contained in 15 independent clones revealed sizes ranging from 150 to 360 bp. We chose the plasmid containing the largest insert, called pPD-3, for further analysis.

Identification of phosphodiesterase cDNA

The nucleotide sequence of pPD-3 was determined by the dideoxy method using the 17-base oligonucleotide as a sequencing primer (Fig. 1). The results of repeated analyses were identical, and revealed a sequence which accounted perfectly for 14 amino acids extending from the primer to the amino-terminal residue determined for the mature phosphodiesterase.

Hybridization selection of poly(A)+ RNA utilizing filter-bound pPD-3 demonstrated that this plasmid selected an mRNA which could be translated into material recognized by anti-phosphodiesterase serum (Fig. 2). This material has an apparent molecular size of 48 kDa, identical to that determined for phosphodiesterase from *in vitro* translation studies utilizing unselected poly(A)+ RNA (Mullens *et al.*, 1984). The combined results of nucleotide sequence analysis and hybrid selection confirm that pPD-3 contains cDNA coding for a portion of the *D. discoideum* phosphodiesterase.

Gene copy number and phosphodiesterase transcript size

Southern blots of *D. discoideum* nuclear DNA cleaved with a series of restriction enzymes added singly or in pairs indicated that in all cases a single band hybridized with the phosphodiesterase-specific probe. Representative results of this analysis are shown in Fig. 3. Using pPD-3 as a calibration standard, we determined that the intensity of hybridization was that expected for a single copy gene. A restriction map of the region surrounding the phosphodiesterase gene was constructed from the analysis of DNA blots and is shown in Fig. 4.

Fig. 2. Hybridization selection. Fluorograph of ^{35}S -labelled products of *in vitro* translation immunoprecipitated with anti-phosphodiesterase serum. Translation was programmed in a rabbit reticulocyte lysate system with poly(A)+ RNA selected by filter-bound plasmid DNA. The plasmids used were as follows: lane 1, pPD-3; lane 2, p08-115, a plasmid containing pBR322 and a randomly selected 2.3 kbp fragment of *D. discoideum* genomic DNA; lane 3, a control filter containing no DNA. Molecular size markers shown correspond to the positions of gamma globulin (52 kDa), ovalbumin (45 kDa) and carbonic anhydrase (30 kDa). The arrow marks the 48 kDa position of phosphodiesterase.

Northern blot analysis indicated a single band, corresponding to an mRNA 2.1 kb in size, which hybridized with the probe for phosphodiesterase (Fig. 5). The minimum length of an mRNA which could encode a protein the size of phosphodiesterase is approximately 1.3 kb, indicating that the phosphodiesterase transcript contains extensive untranslated regions.

DISCUSSION

We have isolated a cDNA clone encoding the amino-terminal portion of the cyclic nucleotide phosphodiesterase of *D. discoideum*. The approach taken for obtaining this clone was to create a cDNA library enriched for phosphodiesterase sequences, but it precluded obtaining a full length copy of this gene. Based on the estimate that a maximum of 0.015% of translatable mRNA encodes phosphodiesterase (Mullens *et al.*, 1984), and the observation that approximately 1 in 200 clones hybridized with the oligonucleotide probes, this procedure enhanced recovery of phosphodiesterase cDNA at least 50-fold above that anticipated for oligo(dT) priming of cDNA synthesis.

Fig. 3. DNA blot of double digests of genomic DNA hybridized with phosphodiesterase-specific probe. DNA (1 µg) was run in each lane of a 0.8% agarose gel, then transferred to Gene Screen Plus (New England Nuclear), and hybridized with ^{32}P -labelled probe according to the manufacturer's instructions. DNA in lanes 1–4 was digested with *Bam*HI, together with *Bcl*I (lane 1), *Hind*III (lane 2), *Pst*I (lane 3) and *Xho*I (lane 4). DNA in lanes 5–7 was digested with *Hind*III together with *Bcl*I (lane 5), *Pst*I (lane 6) and *Xho*I (lane 7). In lanes 8 and 9 DNA was digested with *Pst*I together with *Bcl*I (lane 8) and *Xho*I (lane 9). The position and size of lambda phage *Hind*III restriction fragments are indicated.

Fig. 4. Restriction map of the region encompassing the phosphodiesterase gene. The area represented by the open box is the smallest fragment found which hybridized with the phosphodiesterase probe. Numbers below the map indicate distances in kbp. Not shown on the map are sites for the enzymes *Ava*II, *Bgl*I, *Bam*HI, *Hind*III, *Msp*I, *Pst*I, *Pvu*II or *Sal*I which are located at distances at least 10 kbp from the boxed *Bcl*I–*Eco*RI fragment.

DNA blot analysis demonstrated that the phosphodiesterase gene of *D. discoideum* exists as a single copy in the nuclear genome. Previous results from this laboratory have indicated that the membrane-bound and secreted forms of phosphodiesterase are closely related, if not identical enzymes (Shapiro *et al.*, 1983). We believe that the single gene detected in this study encodes the activity found in both forms of phosphodiesterase.

Sequence analysis of the phosphodiesterase cDNA revealed an open reading frame which extended to the 3' terminus of the cDNA, and which coded for 65 amino acids beginning with a methionine codon. This was the longest open reading frame in pPD-3, and the only one which continued to the 3' end of the cDNA. A codon utilization which strongly favors A or T in any degenerate position is typical for *D. discoideum*, producing an average G + C content of 38% in the translated portions of 15 genes analysed by Kimmel & Firtel (1983). The bias for the use of A or T is especially pronounced in pPD-3, which is characterized by a 21% G + C content in the region extending in the 3' direction from the ATG codon. We believe the methionine codon present in pPD-3 represents the site of translation initiation *in vivo* because: (i) immediately adjacent to this methionine is a region possessing characteristics of a single sequence (Watson, 1984), including a run of 12 hydrophobic amino acids; (ii) initiation from this methionine would account for the size difference observed between purified and deglycosylated phosphodiesterase and phosphodiesterase produced by translation *in vitro*; and (iii) an in-frame TAA stop codon is located 39 nucleotides upstream of this ATG triplet.

Fig. 5. RNA blot hybridization with a phosphodiesterase-specific probe. Poly(A)⁺ RNA (10 µg) purified from cells starved for 3 h in buffer containing 1 mM-cAMP was glyoxylated and separated by electrophoresis through a 1% agarose gel before blotting. The indicated size markers are the 4.1 and 1.8 kb rRNAs of *D. discoideum*. The size of the band hybridizing with probe is 2.1 kb.

The majority of phosphodiesterase in *D. discoideum* is destined for export from the cell, and if this process follows the typical route of transmembrane transport, the presence of a signal sequence in this protein is anticipated. A portion of the derived amino acid sequence proximal to the initiator methionine possesses characteristics of a signal sequence. However, if the entire segment of 49 amino acids extending from the methionine to the amino-terminal codon determined by amino acid analysis acts as a leader sequence, it would be unusually long compared to the 20 to 30 amino acids common for eukaryotic signal sequences (Watson, 1984). We do not know if the phosphodiesterase we have purified is the primary product of a processing event such as signal sequence cleavage, or if this protein is the result of some additional processing events. One striking feature of this sequence is the predominance of acidic residues in the region extending from positions 27 to 35.

The availability of this phosphodiesterase cDNA fragment should allow the isolation of the complete gene from genomic or full-length cDNA libraries, and facilitate studies of the structure and regulation of the phosphodiesterase gene.

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