Molecular cloning of a cDNA coding for GTP cyclohydrolase I from *Dictyostelium discoideum*

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The GTP cyclohydrolase I (GTP-CH) gene of the cellular slime mould *Dictyostelium discoideum* has been cloned and sequenced. The 855 bp cDNA of this gene contains the open reading frame (ORF) encoding 232 amino acids with a predicted molecular mass of approx. 26 kDa. Southern blot analysis indicated the presence of a single gene for GTP-CH in *Dictyostelium*. PCR amplification of the ORF from chromosomal DNA and sequencing showed the existence of a 101 bp intron in the GTP-CH gene of *Dictyostelium discoideum*. The amino acid sequence has 47 % and 49 % positional identity to those of the human and yeast enzymes respectively. Most of the sequence variation between species is located in the N-terminal part of the protein. The overall identity with the *E. coli* protein is markedly lower. The enzyme was expressed in *E. coli* and purified as a 68 kDa fusion protein with the maltose-binding protein of *E. coli*. GTP-CH of *Dictyostelium* is heat-stable and showed maximal activity at 60 °C. The K_m value for GTP is 50 μM.

INTRODUCTION

GTP cyclohydrolase I (GTP-CH; EC 3.5.4.16) is the first enzyme in the biosynthesis of tetrahydrobiopterin (BH_4). It catalyses the conversion of GTP into dihydroneopterin triphosphate [6-(D-*threo*-1′,2′,3′-trihydroxypropyl)-7,8-dihydropterin]. The subsequent action of 6-pyruvoyltetrahydropterin synthase and sepiapterin reductase yields the final product BH_4, which has multiple functions. It serves as an electron donor for the hydroxylation of aromatic amino acids [1–3], is involved in the generation of NO from arginine [4] and serves as a modulator in signal transduction [5,6]. In mammals, the biosynthesis of BH_4 is regulated in a tissue-dependent manner through the activity of the rate-limiting enzyme GTP-CH. The cytokines interferon-γ and kit-ligand were shown to regulate the activity of GTP-CH by increasing the steady-state level of its mRNA [7,8].

The cellular slime mould *Dictyostelium discoideum* grows as single-cell amoebae. In its life cycle, starvation initiates a developmental programme which starts with multicellular aggregation followed by the formation of fruiting bodies containing spores [9]. This process is controlled by cAMP, whose signal transduction is linked to heterotrimeric G-proteins [10,11]. Recently, we have shown that tetrahydrodictyopterin [12,13], the D-*threo*- isomer of BH_4, originates from GTP by *de novo* biosynthesis requiring the action of GTP-CH [14]. During the developmental process the activity of GTP-CH is regulated independently in the cytosolic and membrane fractions. In the cytosol the enzymic activity declines along with the amount of the GTP-CH specific 0.9 kb mRNA, whereas in the membrane fraction the activity culminates 4 h after induction. A G-protein-linked signalling pathway is involved in the regulation of GTP-CH activity and thus of tetrahydrodictyopterin production during the early development of *Dictyostelium* [14]. The cloning and expression of this enzyme should provide the basis for a deeper understanding of the mechanisms involved in the regulation of GTP-CH in *Dictyostelium*, on the genomic level as well as by a post-translational modification of the enzyme.

EXPERIMENTAL

Materials

Restriction endonucleases and modification enzymes were obtained from New England Biolabs (Schwalbach, Germany) or United States Biochemical (Cleveland, OH, U.S.A.). Oligonucleotides were synthesized using an ABI synthesizer, model 394-8 (Applied Biosystems Inc., Weiterstadt, Germany).

PCR and cloning of PCR products

Total RNA was isolated from *Dictyostelium discoideum* as described previously [15]. Prior to reverse transcription, the RNA was preincubated without enzymes for 5 min at 75 °C and placed on ice for 5 min. The reverse transcription reaction was performed in a final volume of 10 μl containing 1 μg of total RNA from *Dictyostelium*, KCl (10 mM), Tricine, pH 8.4 (15 mM), $MgCl_2$ (3 mM), dithiothreitol (10 mM), 0.3 % Tween 20, dNTPs (1 mM), $(dT)_{20}$ primer (1.25 mM), 10 units of RNasin (Promega, Heidelberg, Germany) and 100 units of Moloney murine leukaemia virus reverse transcriptase (Promega) for 60 min at 42 °C. By adding compounds to the reverse transcription reaction, PCR was carried out in a final volume of 50 μl

Abbreviations used: BH_4, (6*R*)-5,6,7,8-tetrahydrobiopterin [biopterin is 6-(L-*erythro*-1′,2′-dihydroxypropyl)pterin]; GTP-CH, GTP cyclohydrolase I; ORF, open reading frame; RACE, rapid amplification of cDNA ends.

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The nucleotide sequence data reported have been deposited in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under accession no. Z49706.

containing KCl (12 mM), Tricine, pH 8.4 (30 mM), $MgCl_2$ (2.6 mM), dithiothreitol (2 mM), 0.3% Tween 20, dNTPs (25 pM), primers (0.4 mM) and 1.5 units of *Taq* DNA polymerase (Promega) for 30 cycles in a thermocycler (Thermocycler 60; Bio-med) (non-recurring denaturation step for 1 min at 96 °C, followed by 30 cycles of 96 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min). Routinely DMSO (5%, v/v) was added to the reaction to prevent unspecific PCR products being formed. The PCR products were separated by electrophoresis in a 1.5% agarose gel, isolated with the Magic PCR Prep kit (Promega), and cloned using the pGEM-T plasmid system (Promega). Labelling of the oligonucleotides was carried out with 135 pmol of the DNA, 25 μCi of [γ-^{32}P]dATP (2.5 μl; Amersham), 2 μl of T4 polynucleotide kinase buffer and 20 units (2 μl) of polynucleotide kinase (New England Biolabs) in a total volume of 20 μl for 60 min at 37 °C.

Isolation of chromosomal DNA of *Dictyostelium* and Southern blotting

Approx. 1×10^7 *Dictyostelium* cells were suspended in 1 ml of lysis buffer (100 mM Tris/HCl, pH 7.8, 5 mM EDTA, 0.2% SDS, 200 mM NaCl) and incubated with proteinase K (100 μg/ml) at 56 °C overnight with agitation. The suspension was vortexed for 30 s and the cell debris was removed by centrifugation (15000 ***g***, 10 min). The supernatant was added to a tube containing an equal volume of propan-2-ol and mixed gently by inversion until a thread of chromosomal DNA was observed. Further purification of the DNA, Southern blotting and ^{32}P-radiolabelling of the cDNA probe were performed as described previously [8,16].

5′ Rapid amplification of cDNA ends (RACE) and 3′ RACE

5′ RACE was performed with the 5′ RACE System (Gibco BRL, Eggenstein, Germany) according to the manufacturer's instructions using the primer 'backrace' (5′-GCCACGCGTCGACTAGTACG-3′) synthesized by us for reamplification in the second and third rounds of PCR. The PCR products obtained by the reamplification were purified and subcloned in the T-tailed pGEM-T vector. White colonies, which were selected on ampicillin/isopropyl β-D-thiogalactoside/X-gal (5-bromo-4-chloroindol-3-yl β-D-galactopyranoside) agarose plates, were hybridized with a nested radiolabelled oligonucleotide. DNA was isolated (Nucleobond; Macherey & Nagel, Düren, Germany) from positive clones and sequenced in both directions.

3′ RACE was performed using the primer backrace T20 [5′-GCCACGCGTCGACTAGTA-CG$(T)_{20}$-3′] for the reverse transcription reaction. For amplification and reamplification of the 3′ cDNA end, the PCR was performed using the gene-specific nested primers together with the backrace primer. The PCR products resulting from the RACE experiments were separated in a 1.5% agarose gel and cloned into the pGEM-T vector. To confirm the specificity of the DNA, the clones obtained were rehybridized overnight at 42 °C with another nested ^{32}P-radiolabelled oligonucleotide. DNA from positive clones was isolated and used for sequencing of the insert.

Sequence analysis

Plasmid DNA purified by ion-exchange chromatography (Nucleobond) was sequenced in both directions using different oligonucleotides. DNA sequencing was performed by the non-radioactive cycle sequencing method using the *Taq* polymerase sequencing kit and dye-labelled dideoxynucleotides on a 373A system (Applied Biosystems Inc.) and a thermocycler (model 9600; Perkin Elmer, Überlingen, Germany). The sequencing reaction was carried out according to the manufacturer's instructions; however, DMSO (5%, v/v) was added to the cycle sequencing reaction mixture to prevent secondary structure formation in the DNA template.

Cloning of a genomic GTP-CH clone and of a GTP-CH expression clone fused to maltose-binding protein of *Escherichia coli*

Based on the DNA sequence yielded by the RACE experiments, the PCR primers 5′-GGATCCATGAGTGACAATTTAAAATCATACC-3′ ('Start') and 5′-CTGCAGTTAATTAGTTGAGTTAAATAAACTGAAGAATTCAGC-3′ ('Stop') were designed. Their positions are shown in Figure 1. PCR experiments using these primers together with either chromosomal DNA or a reverse transcription reaction of total RNA of *Dictyostelium* as template yielded two amplification products of different lengths. These products were cloned in the pGEM-T plasmid. The first 6 bases of the primers (underlined) contained recognition sites for the restriction enzymes *Bam*HI and *Pst*I, and were added in order to facilitate easy transfer of the GTP-CH cDNA from the pGEM-T clone in the pMal-c2 bacterial expression vector (New England Biolabs).

Purification of the (maltose-binding protein)–GTP-CH fusion protein

The GTP-CH coding region was subcloned into the pMal-c2 bacterial expression vector (New England Biolabs) and expressed in *E. coli* strain XL-1 (Stratagene, Heidelberg, Germany). A fresh overnight culture of pMal-c2/GTP-CH-transformed *E. coli* (XL-1) was diluted 1:20 with LB medium containing ampicillin (100 mg/l). The culture was induced with isopropyl β-D-thiogalactoside (2 mM) and grown for 36 h at room temperature. The cells were harvested by centrifugation at 3200 ***g*** for 10 min and resupended in ice-cold lysis buffer (20 mM Tris/HCl, pH 7.0, 0.2 M NaCl). The cell suspension was sonicated and centrifuged at 10000 ***g*** for 10 min at 4 °C to remove bacterial debris. To isolate the fusion protein, the supernatant was incubated with amylose resin (New England Biolabs) according to the manufacturer's instructions.

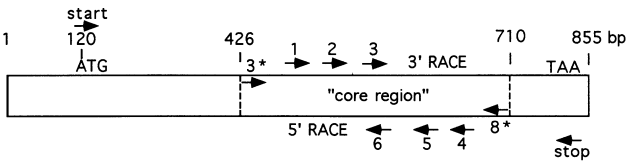
Determination of GTP-CH activity

The purified fusion protein was used for kinetic studies. A 50 μl aliquot of a diluted protein solution (43 μg/ml) was incubated with 15 μl of GTP solution (2 mM) at different temperatures for 1 h. The resulting dihydroneopterin triphosphate was oxidized to neopterin phosphates by treatment with acidic iodine (30 μl; 1% I_2/2% KI in 1 M HCl) for 15 min. Excess iodine was reduced by addition of 15 μl of 3% ascorbic acid. The reaction products were determined fluorimetrically following HPLC under ion-pairing conditions [17].

RESULTS

Cloning and sequencing of *Dictyostelium* GTP-CH

Starting from the core region cDNA of *Dictyostelium* GTP-CH that we had identified earlier by homology cloning [18], amplification of the 5′ end was performed by 5′ RACE using nested primers which were located in the antisense strand and selected from the central part of our cDNA sequence (Figure 1). The sequence obtained encompassed the 5′ end of the open reading



Primer	Position	Sequence (5'-->3')
1	474-498 bp	TCATTATGTGAACATCATATGGTGC
2	503-523 bp	CCATGGTAAATGCCATATTGG
3	571-593 bp	TCGTGAAATTTTTGCCAGAAGG
4	664-641 bp	ACACCCATTGGATTTAAGTACC
5	636-606 bp	TATTTGCCTGGTTAAACG
6	595-573 bp	AACCTTCTGGCAAAAATTTCAGC
start		GGATCCATGAGTGACAATTTAAAATCATACC
stop		CTGCAGTTAATTAGTTGAGTTAAATAAACTGAAGAATTCAGC

Figure 1 Sequence and location of primers used for RACE and PCR experiments

The primers were used to yield the 5′ cDNA end (primers 4, 5 and 6) and the 3′ cDNA end (primers 1–3) from *Dictyostelium* via RACE. Primers 3* and 8* were used to obtain the core region of the cDNA, as described elsewere [18]. The primers Start and Stop were used to obtain a genomic clone and a cDNA clone from *Dictyostelium* GTP-CH, as described in the Experimental section.

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ATATATGTAA ATATAAAAAA AAAAAAAATA AAAAAAAAAA AATAAAACCA   50

AATTAAATAA AAAAAACTAT TCTATAAATC AACAACAATA ATAACATATA  100

CATATACAAA TAAAATAAAA TGAGTGACAA TTTAAAATCA TACCAAGATA  150
                        M  S  D  N   L  K  S   Y  Q  D>

ATCATATTGA AAATGAAGAT GAAGAAATTT ATGAAAGATC AAATGGAAAA  200
N  H  I  E   N  E  D   E  E  I   Y  E  R  S   N  G  K>

GGAAAGGAAT TAGTTGATTT TGGAAAGAAG AGAGAACCAT TAATCCATAA  250
 G  K  E   L  V  D  F   G  K  K   R  E  P   L  I  H  N>

CCATGAAGTT TTAAATACAA TGCAATCATC AGTAAAGACA TTATTAAGTA  300
  H  E  V   L  N  T   M  Q  S  S   V  K  T   L  L  S>

GTTTAGGTGA AGATCCAGAT AGAGAAGGTT TATTAAAGAC ACCATTAAGA  350
S  L  G  E   D  P  D   R  E  G   L  L  K  T   P  L  R>

ATGTCAAAGG CTTTATTATT TTTTACACAA GGTTATGAGC AATCTGTTGA  400
 M  S  K   A  L  L  F   F  T  Q   G  Y  E   Q  S  V  D>

TGAAGTTATT GGTGAAGCAA TTTTTAATGA AAATCATCAT GAAATGGTTG  450
  E  V  I   G  E  A   I  F  N  E   N  H  H   E  M  V>

TTGTCAGAGA TATTGATATA TTTTCATTAT GTGAACATCA TATGGTGCCA  500
V  V  R  D   I  D  I   F  S  L   C  E  H  H   M  V  P>

TTCCATGGTA AATGCCATAT TGGTTATATT CCAGATCAAA AAGTTTTAGG  550
 F  H  G   K  C  H  I   G  Y  I   P  D  Q   K  V  L  G>

TTTAAGTAAA TTAGCAAGAG TTGCTGAAAT TTTTGCCAGA AGGTTACAAG  600
  L  S  K   L  A  R   V  A  E  I   F  A  R   R  L  Q>

TTCAAGAACG TTTAACCAGG CAAATAGCAC AAGCAATTCA AGCTCACTTA  650
V  Q  E  R   L  T  R   Q  I  A   Q  A  I  Q   A  H  L>

AATCCAATGG GTGTTGCCGT CGTAATTGAT GCATCACACA TGTGTATGGT  700
 N  P  M   G  V  A  V   V  I  D   A  S  H   M  C  M  V>

TATGAGAGGT GTACAAAAAC CAGGTGCAAG TACTGCCACC TCATCTGTTT  750
  M  R  G   V  Q  K   P  G  A  S   T  A  T   S  S  V>

GCGGTATTTT TGAAGAAGAC TCTAGAACTC GCGCTGAATT CTTCAGTTTA  800
C  G  I  F   E  E  D   S  R  T   R  A  E  F   F  S  L>

TTTAACTCAA CTAATTAATA ATAATAATTA TAATTAATAA TAAAAAAAAA  850
 F  N  S   T  N  *

AAAAA                                                   855
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Figure 2 Nucleotide sequence and deduced amino acid sequence of GTP-CH from *Dictyostelium discoideum*

The nucleotide sequence of GTP-CH from *Dictyostelium* was obtained from three overlapping cDNA clones generated by 5′ RACE (nucleotides 1–572), by using our degenerate primers [18] (nucleotides 426–710) and by 3′ RACE (nucleotides 582–855). It is presented along with the deduced amino acid sequence (one-letter code), and contains the 5′ untranslated region, the ORF starting from the putative first methionine residue, and a short 3′ untranslated region containing the putative polyadenylation signal AATAAA.

frame (ORF) of *Dictyostelium* GTP-CH and its 5′ untranslated region. By analogy with the 5′ RACE, a 3′ RACE was used to obtain the 3′ part of the ORF (Figure 1). Following reverse transcription of total RNA, two successive PCRs were performed using two nested, gene-specific primers and the 'backrace' primer. The resulting amplificate encompassed about 320 bp (results not shown). This product was cloned and sequenced. The sequence obtained by the 3′ RACE experiments encompassed the 3′ end of the ORF of *Dictyostelium* GTP-CH and its 3′ untranslated region. The use of the backrace primer for both 3′ and 5′ RACE simplified PCR amplification and sequencing. The sequence of the complete ORF (Figure 2) was assembled by combining the sequences of the 5′ end, the central part and the 3′ end. The ORF encodes a protein of 232 amino acids with a predicted molecular mass of 26227 Da. The amino acid sequence of *Dictyostelium* GTP-CH shows 47 % identity to the human and 49 % identity to the yeast GTP-CH sequences, but only 30 % identity to the *E. coli* protein. Manual multiple sequence alignment reveals that the N-terminal part of this enzyme is completely different from that of mammals, insects, yeast and bacteria, whereas the remainder of the protein sequence is very similar in all species (Figure 3).

Heterologous expression of *Dictyostelium* GTP-CH

For the expression of the enzyme, the entire ORF encoding GTP-CH was amplified by RT-PCR using the primers Start and Stop, yielding a PCR product of 710 bp. This PCR product was cloned into the expression vector pMal-c2. The expression of the (maltose-binding protein)–GTP-CH fusion protein yielded a 68 kDa protein detected by SDS/PAGE (results not shown). The fusion protein was purifed by affinity chromatography and showed GTP-CH activity, confirming the correct sequence information obtained by previous RACE experiments. The dependence of the specific activity on temperature is presented in Figure 4. The K_m value for GTP of the purified recombinant protein was 50 μM. The enzyme is heat-stable, and shows maximum activity at 60 °C (Figure 4).

Genomic structure

Southern blot analysis of chromosomal DNA from *Dictyostelium* was performed with the radiolabelled 284 bp cDNA probe, encoding from Glu-104 to Gly-197. It indicated that the gene is present as a single copy (Figure 5). Amplification of the GTP-CH gene from chromosomal DNA was performed using the same primers Start and Stop which were used for amplification of the cDNA coding for the active enzyme. The chromosomal amplification revealed a slightly longer PCR fragment, suggesting the existence of a small intron in the gene of GTP-CH. This was confirmed by sequencing the cloned genomic amplification product. It showed the same 5′ coding DNA sequence as the cDNA, the transition in the intron and the re-entry in the 3′ coding part which was again identical to the cDNA. The DNA

Figure 3 Amino acid sequence comparison with the homologous enzymes from human, mouse, rat, *Drosophila melanogaster*, yeast and *E. coli*

The deduced amino acid sequences of human [19,20], mouse [22], rat [21], *Drosophila melanogaster* [26], yeast (K. Witter, M. Gütlich, R. Stucka, T. Werner, I. Ziegler and A. Bacher, unpublished work), *Dictyostelium* and *E. coli* [30] GTP-CHs are aligned. The amino acid sequence of the *Dictyostelium* enzyme is underlined. Quotation marks ('') represent residues which are identical to the human sequence.

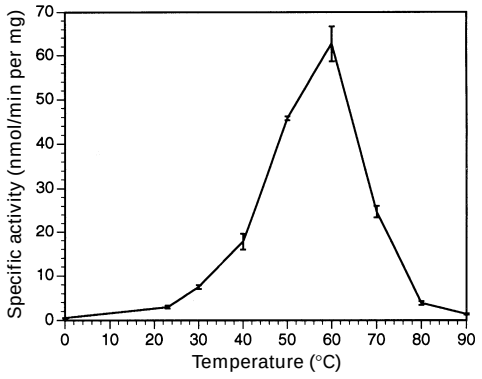

Figure 4 Specific activity of GTP-CH from *Dictyostelium discoideum*: effect of temperature

The enzymic activity was determined by measuring neopterin via HPLC. The values represent means ± S.D. of triplicate determinations.

sequence of the intron of 101 bp between the codons for amino acids Glu-108 and Met-109 is outlined in Figure 6.

DISCUSSION

The ORF of *Dictyostelium* GTP-CH and the 5′ and 3′ untranslated regions were amplified by reverse transcription-PCR and the RACE technique. This ORF, amplified by using specific oligonucleotides derived from the RACE sequence data, coded for a protein with GTP-CH activity when expressed in *E. coli*. The kinetic data for the recombinant enzyme are comparable with those for other eukaryotic GTP-CHs. The enzyme from *Dictyostelium* was shown to be heat-stable, although this organism is thermosensitive and is not able to withstand temperatures above 25 °C. This may reflect the ancient origin of GTP-CH from a putative thermophilic ancestor, with the properties of the enzyme being maintained during evolution.

The deduced protein sequence of *Dictyostelium* GTP-CH showed strong similarity to those of the enzymes from human [19,20], rat [21] and mouse [22], as well as to the yeast protein [28]. The identity with the bacterial enzyme is markedly lower. The N-terminal part of the enzyme, which differs between species, may be involved in species-specific protein–protein interactions, which are, for example, responsible for the post-translational regulation of GTP-CH activity in *Dictyostelium* [14]. The recent finding that tetrahydropterins interfere with G-protein-linked signalling pathways [24] further supports the hypothesis that GTP-CH participates in these regulatory mechanisms. It has been shown [14] that in *Dictyostelium* GTP-CH exists in both cytosolic and membrane-associated forms. The N-terminal sequence of *Dictyostelium* GTP-CH does not include a transmembrane region or a peptide sequence that would render post-translational modifications to allow membrane anchoring. Therefore the unique sequence of the N-terminus may be responsible for a protein–protein interaction with a membrane-bound protein. Furthermore, it was

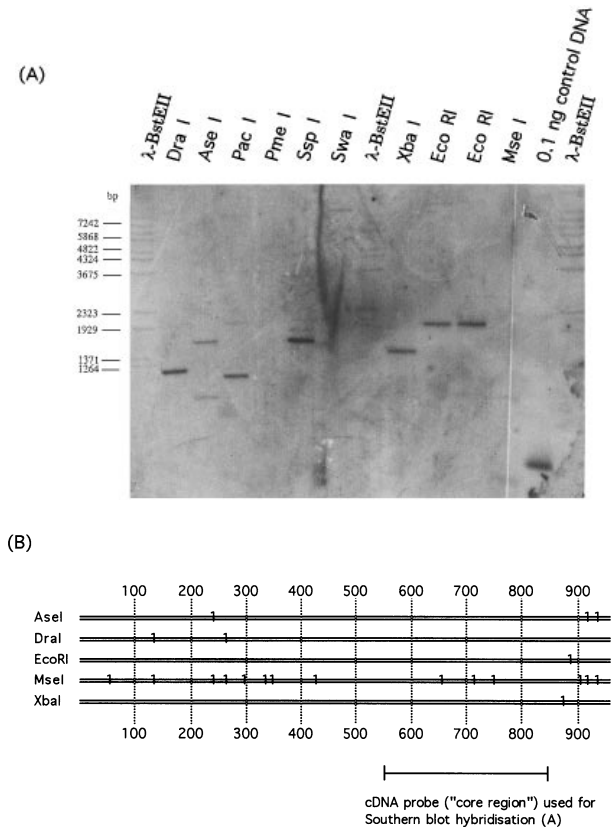

Figure 5 Southern blot analysis and restriction map of the GTP-CH gene of *Dictyostelium discoideum*

(**A**) Chromosomal DNA from *Dictyostelium* was digested with the restriction enzymes indicated, separated on a 1% agarose gel and blotted on to a membrane. The DNA was hybridized with a 284 bp ^{32}P-labelled cDNA probe specific for GTP-CH. (**B**) The indicated restriction enzymes were used for digestion of chromosomal DNA for Southern blot analysis and showed one or more cleavage site(s) in the GTP-CH gene. The other restriction enzymes (*Pst*I, *Pac*I, *Pme*I, *Ssp*I and *Swa*I) have no cleavage site in this region. The larger fragment recognized by the probe in the *Ase*I lane may result from incompletely cleaved chromosomal DNA.

shown for the *E. coli* enzyme [25] that the N-terminal part of GTP-CH is located on the surface of the decameric protein. The amino acids relevant for catalysis and structural stability are conserved between *Dictyostelium* and *E. coli* [18]. The elongated N-terminus of *Dictyostelium* GTP-CH may also be located at the surface of the multimeric enzyme, allowing the formation of a domain that interacts with other proteins.

In contrast to the yeast enzyme, the gene encoding GTP-CH in *Dictyostelium* contains one intron (Figure 6). Interestingly, this splice site is not at the same position as those in the GTP-CH genes of the higher eukaryotes *Drosophila melanogaster* [26], mouse and human [27]. The sequence of the *Dictyostelium* gene in the region corresponding to the splice site in these species does not allow splicing; in addition, the sequences of the higher eukaryotes cannot be spliced at the position of the *Dictyostelium* intron. This suggests that *Dictyostelium* has branched off from other animal species very early in evolution, but after separation of the animals from the fungi. Recent evidence shows that, in contrast to the single mRNA detected in *Dictyostelium*, man [8] or yeast [28], there are two specific mRNAs present in rodents [19] and chicken (results not shown).

Our previous data [14] showed that the concentration of mRNA specific for GTP-CH depends markedly on the life cycle of *Dictyostelium* and that, after initiation of development, the mRNA level decreases within 2 h to about 15% of the initial value. Further analysis of this gene and investigations of the observed rapid down-regulation of the GTP-CH mRNA level during the developmental process may shed light on as yet unknown mechanisms of negative gene regulation in *Dictyostelium*.

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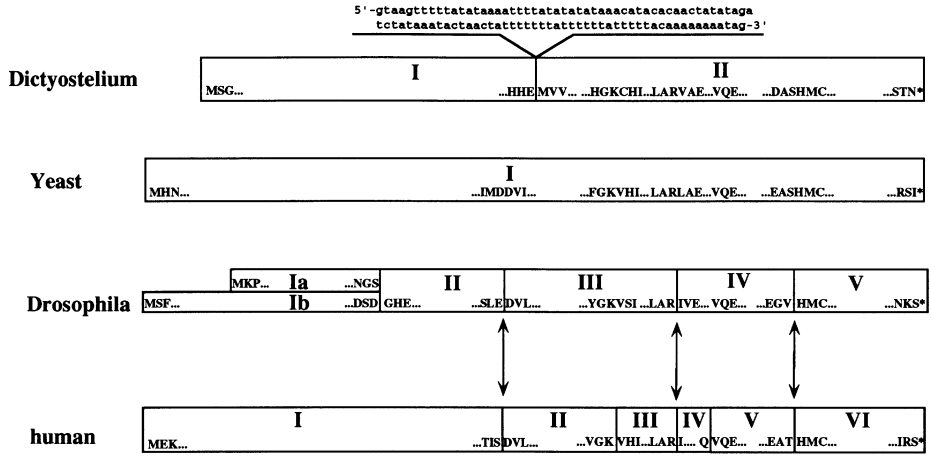

Figure 6 Comparison of the genomic structures of GTP-CH from *Dictyostelium discoideum*, yeast, *Drosophila melanogaster* and human

The *Dictyostelium* gene is split into two exons by a 101 bp intron situated between the codons encoding Glu-108 and Met-109, as shown by PCR using genomic DNA as a template. The data were confirmed by sequencing of the genomic clone. The exon/intron boundary of the *Dictyostelium* gene conforms to the GT/AG rule [31], but it is not located at the same position as the comparable transition that is conserved in the higher eukaryotes *Drosophila*, human and mouse.

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