Molecular cloning and cell-cycle-dependent expression of the acetyl-CoA synthetase gene in *Tetrahymena* cells

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To identify transcriptionally regulated mediators associated with the cell cycle, we adopted the differential mRNA display technique for cell cultures of *Tetrahymena pyriformis* synchronized by cyclic heat treatment. One cDNA fragment that was expressed differently during synchronous cell division had a greatly decreased expression at 30 min after the end of heat treatment (EHT). Using this fragment as a probe, we isolated the full-length cDNA for *T. pyriformis* acetyl-CoA synthetase (*TpAcs*) which encodes a 651 amino acid polypeptide with a predicted molecular mass of 72.8 kDa. The deduced amino acid sequence of *T. pyriformis* ACS shows 42% sequence identity compared with that of *Lysobacter* sp. acetyl-CoA synthetase (ACS), an enzyme which catalyses the formation of acetyl-CoA from acetate via an acetyl-adenylate intermediate. The deduced sequence is also 41% and 40% identical compared with those of *Pseudomonas putida* and *Coprinus cinereus* ACS, respectively. The

deduced sequence of *T. pyriformis* ACS also shares similar characteristics of the conserved motifs I and II in the ACS family. To further investigate the actions of the gene encoding this enzyme, mRNA expression was determined during the course of synchronized cell division in *T. pyriformis*. Northern blot results show that the mRNA level was dramatically decreased at 30 min after EHT prior to entering synchronous cell division (which occurs 75 min after EHT), suggesting that mRNA expression of the *TpAcs* was associated with the cell cycle and that the down-regulated expression of *TpAcs* at 30 min after EHT would be required for the initiation of the oncoming synchronous cell division in *T. pyriformis*.

Key words: synchronous cell division, differential display, acetate activation, two-component regulatory system.

INTRODUCTION

The unicellular ciliated protozoan *Tetrahymena* is widely used as a model system for studies on morphogenesis, conjugation, cell division, growth kinetics, membrane biogenesis and membrane adaptation. We have done a series of experiments related to these processes, especially on the molecular basis of cryo-adaptive regulation of membrane lipids. To investigate further the signal-transduction pathway that mediates the stress responses in *Tetrahymena pyriformis*, we have successfully cloned two cDNAs encoding the stress-responsive mitogen-activated protein kinase (MAPK) and the 'never in mitosis in *Aspergillus nidulans*' (NIMA)-related protein kinase (TpNrk) by the mRNA differential display technique [1,2]. This procedure is a PCR-based technique, which is a potential tool for identification of genes differentially expressed among various tissues or cell lines or under different conditions [3]. The deduced sequence of the stress-responsive MAPK from *T. pyriformis* shows significant similarity to other MAPKs, especially p38 and stress-activated protein kinase (SAPK), which are involved in many stress responses [4–6]. TpNrk has high sequence similarity to other Nrk, which function as regulators of the cell cycle [7,8]. We provided evidence that mRNA expression of TpNrk was not only up-regulated in response to stress, but also, interestingly, it was under cell-cycle control thus suggesting its possible role in cell-cycle progression [1]. However, the molecular mechanisms of cell-cycle regulation in *Tetrahymena* cells are still largely

unknown. To further decipher the spectrum of molecular factors, known or unknown, that are involved in the control of the cell cycle, we have attempted to isolate other genes which are differentially expressed during the synchronized cell cycle of *T. pyriformis*.

Using the mRNA differential-display approach, we have identified several cell-cycle-associated transcripts in *T. pyriformis* cells. One of these transcripts shows high sequence similarity to an mRNA, which encodes acetyl-CoA synthetase (AMP forming) (EC 6.2.1.1.) (ACS), a broadly distributed acetate-activating enzyme [9–15]. To our knowledge this is the first demonstration of the possible involvement of ACS in the regulation of cell-cycle progression. Here, we describe the isolation and cell-cycle-dependent expression of the *T. pyriformis* *acs* gene (*TpAcs*) that is structurally related to *acs* genes in other species.

EXPERIMENTAL

Materials

Restriction enzymes and other nucleic acid modifying enzymes were obtained from Boehringer Mannheim, Toyobo (Osaka, Japan) and Nippon Gene (Tokyo, Japan). *Taq* DNA polymerase was from Takara Shuzo (Shiga, Japan). Radiolabelled nucleotides, a sequenase version 2 DNA-sequencing kit and a multiprime-DNA-labelling system were purchased from Amersham. Anchored primers (GT15MG and GT15MA) and

Abbreviations used: ACS, acetyl-CoA synthetase; *acs*, gene or cDNA encoding an ACS; AP, arbitrary primer; EHT, end of heat treatment; MAPK, mitogen-activated protein kinase; Nrk, NIMA-related protein kinase; *TpAcs*, gene or cDNA encoding the *T. pyriformis* ACS; TpNrk, Nrk from *T. pyriformis*.

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The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases under accession number AB026298

arbitrary primers (AP-1–10) were obtained from Genhunter (Nashville, TN, U.S.A.). The TOPO TA Cloning kit was purchased from Invitrogen. GeneScreen Plus hybridization membranes were from DuPont/NEN. *Escherichia coli* strain XL-1 blue was used as the bacterial host for pBluescriptII recombinant plasmids.

Cell culture

For induction of the synchronous cell division, *T. pyriformis* strain W was grown in proteose-peptone medium [1.0% (w/v) proteose peptone, 0.5% (w/v) yeast extract, 0.87% (w/v) glucose] at 26 °C with shaking (90–100 strokes/min). Cells in the early exponential phase were subjected to eight cycles of heat treatment (26 °C for 30 min followed by 34 °C for 30 min for each cycle) as described by Watanabe [16]. To assess the morphological changes and the division index during the synchronization, cells were stained with the DNA-specific dye Hoechst 33258 and were observed by fluorescence microscopy (Olympus BX60, Tokyo, Japan). To verify the effect of carbon source on the expression of the *TpAcs* gene, cells were cultured in PY medium [1.0% (w/v) proteose peptone and 0.5% (w/v) yeast extract], PY medium containing 0.87% (w/v) glucose (PYG medium), and PY medium containing 0.1% (w/v) sodium acetate (PYA medium). The cultures were harvested at 24, 36, 48 and 60 h after inoculation with 1% of their volume of a stock culture.

RNA extraction and differential display analysis

T. pyriformis cells were harvested at 0, 30, 75, 90 min after the end of heat treatment (EHT) and total RNAs were extracted by the guanidinium thiocyanate method [17]. To ensure that the RNA samples were free of contaminating-genomic DNA, they were treated with 1 unit of RNase-free DNase per 10 µg of RNA for 30 min at 37 °C. For differential-display PCR, reverse transcription and PCR, the recovery and re-amplification of cDNAs obtained were carried out essentially as previously described with slight modifications [1,2,18]. Briefly, reverse transcriptase products from 25 ng of total RNA were amplified by PCR, and the amplified cDNAs were separated on 6% (w/v) acrylamide denaturing-sequence gels containing 6 M urea and then subjected to autoradiography. cDNA bands of interest were excised from the gels and eluted by boiling. The eluted DNA was re-amplified by PCR using the same set of primers as for the original differential-display PCR (see the Results section).

Screening of the cDNA library and DNA sequence analysis

Partial cDNA fragments of interest were subcloned into pCR II vectors. One of the cDNA fragments tentatively designated as CC12 (cell cycle 12) was subjected to further analysis. A *T. pyriformis*-λgt10-cDNA library [19] was screened with the subcloned PCR-derived-cDNA fragment as a probe. Plaque hybridization was performed as previously described [1,2]. The cDNA insert of a positive phage clone was released from the λgt10 vector by digestion with *EcoRI* and then subcloned into the pBluescriptII plasmid. Size-fractionated unidirectional deletion of the insert was performed using exonuclease III and mung bean nuclease. The nucleotide sequence was determined by the dideoxy nucleotide chain-termination method [20]. Sequence similarity searches were performed using the BLAST algorithm [21] from the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health (Bethesda, MD, U.S.A.). Nucleotide and amino acid sequence analyses were performed with DNASIS software (Hitachi).

Amino acid alignment was performed using the computer program ClustalX [22].

Southern and Northern blot analyses

Genomic DNA was isolated from nuclei of *T. pyriformis* strain W cells according to the method described by Gall [23]. DNA (10 µg) was digested with restriction endonucleases (*Bgl*II, *Xba*I, *Bam*HI, *Kpn*I, *Pst*I and *Sal*I) and the resulting fragments were subjected to electrophoresis on 1% agarose and transferred to a GeneScreen Plus membrane. The membrane was then hybridized with the mid-*EcoRV*, ³²P-labelled fragment of the clone (nucleotides 928–1612) as a probe. The blots were then visualized by exposure to Kodak X-Omat film. For Northern blotting, samples of total RNA (20 µg) were fractionated on a 1.0% formaldehyde denaturing agarose gel and transferred to a GeneScreen Plus membrane. Northern hybridization was performed as previously described [1,2], and the density of each band was measured using a densitometer (Densitograph Atto, Tokyo, Japan).

RESULTS

Identification of the cell-cycle-associated clones by mRNA differential display

Hoechst 33258 staining revealed that approximately 85% of the cells enter synchronous cell division at 75 min following the 8-cycle heat treatment (data not shown). RNAs were extracted from *T. pyriformis* cells at 0, 30, 75 and 90 min after EHT. These RNA samples were subjected to differential display analysis using ten arbitrary primers (AP-1–AP-10) and two anchored primers (GT15MG and GT15MA). Approx. 1000 bands ranging from 300–700 bp were amplified. The pattern of amplified cDNAs was, for the most part, similar, but several bands showed different intensities. A cDNA fragment (CC12, approx. 600 bp), amplified

Figure 1 Differential mRNA display and Northern blot of a differentially expressed cDNA fragment

(A) Autoradiogram of a typical differential display. A candidate cDNA (CC12) which demonstrates altered expression by differential display is marked by an arrow. (B) Northern blot using a ³²P-labelled cDNA fragment (CC12) obtained by differential display (indicated by the arrow). 20 µg of total RNA were subjected to Northern blot analysis.

Figure 2 The structure, nucleotide and deduced amino acid sequences of *TpAcs* cDNA

(A) Restriction map of the composite *TpAcs* cDNA. The restriction endonuclease sites are indicated. X, *Xba*I; S, *Sal*I; K, *Kpn*I; E, *Eco*RV; H, *Hind*III; P, *Pst*I. (B) The sequence of the composite *TpAcs* cDNA is shown with the sequence of the expected translation product. The single letter amino acid designation is used. The translation termination codon (TGA) is indicated by an asterisk. The putative polyadenylation signal is underlined.

TpAcs	-----MSQHKNAYEIAAYERATNDKTR	23
LsAcs	-----MRVEEFHRRSIEQP--EA	16
PpAcs	-----MSYSSGLIRPDG-----AKKEDRYLNLRYHSVDDP--ES	32
CaAcs	-----MPEST---QQSHLSLDHEKMQQPFKGFTERS---TKPNLADFETYQKLYKQSIENP--NE	53
AtAcs	MSSNSLRHVESMSQLPSGAGKISQLNAVVLGESLASENDLVFPSPKEFSGQALVSSPQQYMEMHKRSMDDP--AA	73
TpAcs	FWRDQTYL-VDFFKKPEVILDRTHP----NPGFWRFFKDSRYNMCMNAVDSHVGELG-NKFAHWHVSGYEGVEK	91
LsAcs	FWAEQARA-IHRTTPQRILDYSN-----PPFRQMFAGGETNLQYNALDRHLDERG-DQLALVTISSETGQTR	82
PpAcs	FWAEQAQR-IHSHVQQRILEASN-----PPFRQMFVGGQTNLSNALDRHLPERA-EQLALVAVSSETGVTQ	99
CaAcs	EFTQAKENLDFFKPPDLARFPVDPKDDYKNGDLPAWFIINGQINACYNALDRWAIKNP-DKPAI IYEGDEPDQGR	127
AtAcs	FWSDIASE-FYFKQKWGDQVFSN-LDVRKGPISTIEFFKGGITNIDYCNCLDKNVEXLGDKTAIHWEGNELGVDA	146
TpAcs	TYTWAELLDNMSRLAGVLRKLGVRGDRMIIMFMVPEAVFGMLACARIGAIHSVVFGGFAAKELSGRIQCKPA	166
LsAcs	ELSYRQVHRBNNVFASAIRALGVGRDVRVIMNMABAVFAMACARIGAVHSVVFGGFAAHNLALRIDDATPK	157
PpAcs	EITYAQLYREYNTFAAVLKRNLVEKGRVIMNMABAVFAMACARIGAIHSVVFGGFAARNLSRIRIDDSQPK	174
CaAcs	IITYGELLKQVSKLAQALTKLGKKGDSNAVLEMIPEAVITLLAIVRIGAMHSVVFAGFSSASLRIRILADSR	202
AtAcs	SLTYSELLQRCQLANLYKDNCKKGDATVITLMLMELPIAMACARIGAVHSVVFAGFSSADSLACRIWCKPN	221
TpAcs	LILGASYGFEPNKVINNYKTILEBAITLGGVP---GIKVLQQR---GDKMTGPVKVGRDFDYHSLMLQAE--RVD	233
LsAcs	LLICADAGMRGGKVIPYKPLVDCAAEQASP---PPHVLVSR---GLDPDLRLIPGRVDYAAALRTQHEDAQVE	226
PpAcs	LLITADAGMRGGRIIPYKPLVDCAAESES---PPKVLIVSR---GLQPELTWVPGREDEYATLRAAVGDAQVS	243
CaAcs	IVITADESKRGKTIETKKIVDALKCEPK---VRNVIVFKR---TGNSHVFPSPGRDLWWHDEMAKYG-PYFP	269
AtAcs	VILTCNAVKRGPKTINLRAIVDAALDQSSKDGVSIGICTYDNLSTATTRENTKWQNGRVDVWQDVISQYP-TSCE	295

TpAcs	CVPVEGDHPLYLYTSGTTGQPKGIVRDTASTCVASSWNMQHINDIHKGDVYFSGSDTGWVGHQPIVYGPLLRG	308
LsAcs	VEWLESNEPSYLLYTSGTTGQPKGVQRDVGSYAVGLALSMWAIYDIRAGQVMFSTSDVGMVAVGHSYNNYGPLIAG	301
PpAcs	VEWLESNDPSYLLYTSGTTGQPKGVQRDVGSYAVVALSMETVFDVAPGQVMFSTSDVGMVAVGHSYNNYGPLIVG	348
CaAcs	PVPVNSDPLFLLYTSGTTGQPKGVQHNTAGYLLGAVLTTKYTFDVHEDDILFTAGDTGWITGHYTCVYGPLLAG	344
AtAcs	VEWVDAEDPLFLLYTSGTTGQPKGVLTHTGSIYMIYTATTPKYAFDYKSTDVYWCATDCCHITGHSYVTYGPLMNG	370
Motif I		
TpAcs	ATITLYEGKPTGTDPGQFWRLEIKKKKGLYTPALRAIRKDDLNGDWIKKFDISSLTNISMAGERCDVPTYE	383
LsAcs	ATSVLYEGLEPTN-PDPGVWNSICERYGRTMFSSPTGIEVLKKQ---DAAWLKKHDLNALHWFLLAGEPLDEPTAH	373
PpAcs	ATSVLLYEGLEPTN-PDAGIWSLSCERYGRTMFSSPTAVRVLKRF---NTDAIARHDLNALRYLFLAGEPLDEPTAN	420
CaAcs	ATSVVFEGTPAY-PNYSRYWEIVDKKKVQPYVPTALELLKRA---GTKYVEKYDLSSERVLGSGVGEPIAEVWH	416
AtAcs	ATVVVLGAGPNY-PDPGRCDIVDRKKKSIFYTPILVRLMRD---DDKFVTRHSRKSRLVLGSGVGEPIINPSAWR	442
TpAcs	MIQKAVPV---LINDNYQTEFGWIIISCNKYNLYTHEVVRPGTAIKAPSFVDVRIIDQNNNEILEP-NKLGRICIK	454
LsAcs	MITDGIGK---TVINDNYQTEFGWVPLALMPGLDLKPVKFGSPGLEMPGYHLRVIDEATGRDVGP-NEKGVLTIV	444
PpAcs	MSRALGK---PVIDNYQTEFGWPAITLHPGLEMKPVKLSAGLENLSYRIKVIDELSGAPVAA-HQKQMLVVE	491
CaAcs	PYNDNIGRGQAHIWDTYQTESGSHLLTLAG--ITETKPGSASLEFFGVDPKILDPPTGEELPDNDVEGVLAIK	489
AtAcs	WFFNVVGDSCRPISDTWQTEFGFMITPLPG--AWPQKPSATPEFFGVQPVIVL-EKGNEIEG-ECSSYLVCVK	513
TpAcs	LPMPSFMLTLYNDEAFIAKLADSPGYLV-GEISGYFDEDRYLNVMARIIDIIINTAGHRLSTAAMEEALLKHP	528
LsAcs	PPLPFGCLSTVWNDDARFLTSFHFHFKELLYSSLDWAIRDEGYTFILGRDQDVINVAGHRLGPREIEESVASHA	519
PpAcs	PPLPFGFMTTIWRDDRFCSSTFPGHFKEQVYSSLDWAQDEGYTYILGRDQDVINVAGHRLGPREIEESAAEAS	566
CaAcs	SAWF-SITRTGIYNDYRNFIDTALPYANYYS-GEAARDRGFYWILGRVDVVMVNSCHRLSTAETIAALIEHP	562
AtAcs	GSWF-GAFRTLFGDHERYETTFPKPPAGYYFS-GECSRKKGYYWLTGRVDVINVSHRIGTAEVESALVLHP	589

TpAcs	NIVEAAVAVAKVDDPKSEIPIGFVVAKTG-PIKDLKAFEKECTAVVRKEIGPVASFNNCILVDKLPKTRSGKILR	602
LsAcs	SVABAAVIGVHDELKQVVPVVFATLKAGDAHP--HAVATGMQDCVVRQLGAVARPARVYVVAALPKTRSGKLLR	592
PpAcs	RVABAAVIGVPEIKQVVPVAVTTLRKTASDELQRREAAAEIRQAMESRLGAVARPARVYIVNLLPKTRSGKLLR	641
CaAcs	IVGSAVWGYADELTQAVAAVSLKDKKAVGEDVENIKKEMILTURKEIGPFAAPKMILLVDDLKPKTRSGKIMR	637
AtAcs	QCABAAVVGIEHEVKSGGIYAFVTLLEG--VPYSEELRKSLVLMRNQISAFAPDRIHWAPDLKPKTRSGKIMR	661
Motif II		
TpAcs	* QTLKKIVDGEKIE-KIPPTIDDAVPAIITVDVNYGLLKKKPTLPQPKL	651
LsAcs	RSIQALAESRD--GDLSTLDDPGALEEVRRALERADAGG-----	631
PpAcs	RSLQALALQADP--GDLSTLDDPSALDEIRHALRRGPDAGN-----	649
CaAcs	RILRKVLAGEEDQLGDISLTPNPGVVQI I DVVHHAKK-----	675
AtAcs	RILRKIASRQLEELGDTSTLADPSVVDQLIALADV-----	713

Figure 3 Amino acid sequence alignments of *T. pyriformis* Acs with Acs from other species

The amino acid sequence of TpAcs was compared with those of *Lysobacter* sp. ACS (LsAcs) (accession number Y07914), *Pseudomonas putida* ACS (PpAcs) (accession number U24215), *Candida albicans* ACS (CaAcs) (accession number AL033502) and *Arabidopsis thaliana* ACS (AtAcs) (accession number AF036618), by using the computer program ClustalX [22]. Gaps were introduced to maximize the sequence similarity. Positions where the amino acid residues are identical in all five organisms are shown with white letters against a black background. The two conserved motifs I and II are indicated by asterisks above the sequences. The amino acid sequence numbers are shown on the right.

with GT15MG and AP-6, recognized an approx. 2.2 kb mRNA whose expression was dramatically decreased at 30 min after EHT. This time point is prior to the onset of synchronous cell division, which occurs 75 min after EHT. This cDNA fragment

was re-amplified and used as a probe in a Northern blot to assess the differential display results, indicating that the mRNA expression of this clone was associated with the cell cycle (Figure 1). Thus, clone CC12 was selected for further analysis.

Figure 4 Southern blot analysis of *TpAcs*

T. pyriformis genomic DNA (10 µg) was digested with restriction enzymes as indicated, and the fragments were separated by electrophoresis on a 1.0% agarose gel. The membranes were hybridized with a ³²P-labelled *EcoRV* *TpAcs* fragment (nucleotides 928–1612) as a probe. *EcoRV/HindIII*-digested λ DNA was used for molecular size standards, which are marked (in kbp) on the left of the figure. O, origin.

Cloning of the full-length cDNA

To obtain the full-length cDNA, the *T. pyriformis*-λgt10-cDNA library was screened by using the CC12 cDNA fragment as a probe. Five positive clones were obtained from approx. 120 000 plaques. The inserts from these clones were subcloned into the *EcoRI* sites of the pBluescript vectors, and the sequence of the longest insert was determined on both strands in opposite directions by the dideoxy nucleotide chain-termination method. However, this clone (1750 bp) did not contain the full-length cDNA. Therefore we attempted to screen the *T. pyriformis*-λgt10-cDNA library directly by PCR. The primers included a specific reverse primer designed from nucleotide sequence 550–574 and a λgt10 reverse or forward primer to obtain the 5'-end. The PCR fragments obtained were not homogeneous, and the longest fragment contained –96 bp from the first ATG, which partly overlapped with the 1750 bp fragment and contained an additional 5'-coding region, extending the sequence from 1750 to 2203 bp. As shown in Figure 2, the overall cDNA contained 2203 nucleotides with a putative open reading frame which encodes a putative protein of 651 amino acids with a predicted molecular mass of 72.8 kDa. The cDNA also contained a 5'-untranslated stretch of 96 nucleotides and a 3'-untranslated region of 150 nucleotides followed by a polyadenylation signal (TTATAAA) at the 3'-end; the last 601 nucleotides are identical to the fragment obtained by differential display PCR. Sequence analysis (DNASIS) reveals that the coding region (1956 bp) has an A + T content of 56.49%, whereas the 5'-upstream (96 bp) and 3'-downstream (150 bp) noncoding regions possess A + T contents of 70.8% and 79.5% respectively, values which are characteristically higher than that in the coding region. These results are consistent with the values calculated for the overall genome of *T. thermophila* [24], indicating that the cDNA we cloned contained a complete coding region.

Figure 5 Changes in mRNA levels of *TpAcs* during synchronous cell division

Cells in the early exponential phase were subjected to a cyclic heat treatment as described in the Experimental section. Total RNA samples (20 µg), taken at the indicated times following EHT, were separated by electrophoresis on a denaturing gel and transferred to nylon hybridization membranes. The membranes were hybridized with a ³²P-labelled *EcoRV*-*TpAcs* fragment (nucleotides 928–1612) as a probe. Bands were detected by autoradiography, and the densities of bands were monitored with a densitometer (Atto Densitograph, Tokyo, Japan). (A) A typical autoradiogram from one of three different cultures is shown. (B) The results shown (-fold increases from the control value) are means ± S.D. (three different cultures). Division indices at indicated times after EHT are shown as %. The Time-after-EHT scales are identical for A and B.

Deduced amino acid sequence comparison

A search of the EMBL and GenBank databases with the sequence confirmed that the cDNA encodes a novel protein and also revealed that the predicted amino acid sequence is highly similar to the ACS from other species [9–15]. Thus we designated this clone as *TpAcs*. We aligned our sequence with other sequences referenced as ACS or homologues from different organisms (Figure 3). Two highly conserved regions were found in the sequence alignment of the ACS family. Motif I LYTS(G/T/S)TG(K/Q)PKG is very rich in Gly, Ser, and Thr and represents a signature of proteins of the AMP-binding family [25], which includes a diverse group of proteins with similar mechanisms of catalytic activity. This activity involves the reaction between ATP and a carboxylate substrate to form an acyl-adenylate, as well as the catalytic transfer of the acyl moiety from acyl-adenylate to an acceptor [26]. Motif II, located at the C-terminus, is LPKTRSGK(I/L)(L/M)R(R/Q). Although the functional significance of motif II is unknown [15], this region can also be regarded as one of the structural characteristics of the ACS family. Among the ACS family, the distance between motif I and motif II was relatively constant, from 107–113 amino acids. A Southern blot of *T. pyriformis* genomic DNA, digested with different restriction endonucleases, was hybridized with a fragment from the *TpAcs* cDNA, then washed under moderately stringent conditions. The restriction map and sequence of this *TpAcs* fragment are in agreement, and when compared with the Southern blot, suggest that there is a single copy of the *TpAcs* gene in *Tetrahymena* (Figure 4).

Functional expression of *TpAcs*

To further explore the function of the *TpAcs* gene, mRNA expression was determined during the synchronous cell division.

Figure 6 Effects of carbon sources on the expression of *TpAcs*

Cells were cultured in proteose peptone–yeast medium containing: no supplementary carbon source (PY medium), 0.87% (w/v) glucose (PYG medium) or 0.1% (w/v) sodium acetate (PYA medium). The cultures were harvested at the indicated times after inoculation with 1% of their volume of a stock culture. The mRNA levels of *TpAcs* were determined by Northern blot analysis as described in the Experimental section. A typical autoradiogram from one of two different experiments is shown.

RNAs were extracted from cells harvested at 0, 15, 30, 45, 60, 75, 90, 105 and 120 min after EHT, and were subjected to Northern blot analysis by using an *EcoRV* fragment of *TpAcs* as a probe. A single band of about 2.2 kb was recognized by the radiolabelled *TpAcs* fragment. Quantification of *TpAcs* mRNA by densitometry showed that there were prominent fluctuations in the level of the transcript through the cell cycle; the amount of *TpAcs* mRNA decreased to 10% of its original level 30 min after EHT, and then gradually increased thereafter (Figure 5). These results are consistent with those of differential display and suggest that the changes in the mRNA level of *TpAcs* are related to the cell cycle.

To test for the effects of carbon sources on the expression of the *TpAcs* gene, the cells were grown in media supplemented with acetate, glucose or no additional carbon source as described in the Experimental section. At various times, cells were harvested, and RNAs extracted from these cells were used for Northern blot analysis. The results show that the mRNA level of the *TpAcs* was profoundly affected by the carbon composition of the growth media. The expression of *TpAcs* was greatly induced by a low concentration of acetate (0.1%), whereas there was no obvious effect of glucose (0.87%) (Figure 6).

DISCUSSION

Cell division is controlled by a complex network of biochemical signals that are similar in all eukaryotic cells. Together, these signals regulate specific transitions in the cell cycle. The best-characterized transitions are those from G1 to S and from G2 to mitosis. Some evidence showed that reversible protein phosphorylation plays a key role in controlling progression through the cell cycle [27,28]. In *Aspergillus nidulans*, initiation and exit from mitosis require the activation of two cell-cycle-regulated protein kinases. One, the p34^{cdc2}/cyclin B histone H1 kinase, is a universal regulator of mitosis in organisms ranging from fungi to humans, and much is known about its regulation through the cell cycle [29,30]. The second kinase, called NIMA, has also been implicated in controlling entry into mitosis, although it is distinct from p34^{cdc2}, structurally as well as biochemically [31,32]. However, the exact role of NIMA-related kinases in cell-cycle regulation remains to be established. In ciliates, cell-cycle progression has been thought to involve p34^{cdc2} homologue(s) and a cyclin-like function has been suggested in

Tetrahymena cells, although one has not yet been identified [33]. Recently, we cloned a cDNA encoding an Nrk, and also provided evidence for its possible role in *Tetrahymena* cell-cycle control [1].

In the present study, we have successfully isolated a cell-cycle-associated cDNA (*TpAcs*) that belongs to the *acs* family according to our database analysis. *T. pyriformis* ACS shares, at the amino acid level, the highest sequence identity (42%) with ACS of *Lysobacter* sp., an archetype of eubacterial organisms, 41% with *Pseudomonas putida* ACS and 40% with *Coprinus cinereus* ACS (Figure 3). The *T. pyriformis* ACS sequence presented here also shares the similar two conserved sequence motifs I and II of the ACS family [15,25]. Two different types of ACS have already been described, both of which have very important roles in the core metabolism of the cell. The enzyme present in many eubacteria and eukaryotes, ACS (AMP forming; EC 6.2.1.1), is an enzyme that first converts acetate and ATP into an acetyl-adenylate intermediate and then transfers the activated acetyl moiety to CoA forming acetyl-CoA [9–11]. *Giardia lamblia*, an amitochondriate eukaryote, contains ACS (ADP forming; EC 6.2.1.13), an enzyme known only from one other eukaryote (*Entamoeba histolytica*) and a few anaerobic prokaryotes; it is responsible for acetate production accompanied by ATP generation [34]. The two enzymes differ in kinetics, are located in different subcellular compartments, and are immunologically different. Brown et al. hypothesized that the ACS pathway functions as a catabolite-repressible, acetate-inducible, high-affinity acetate uptake system that scavenges acetate present extracellularly at relatively low concentrations [35].

In addition to its role in acetate metabolism, it was of great interest to note that the acetate-activation pathway has been implicated in the regulation of signal transduction by two-component regulatory systems in several bacterial species [36–38]. In *E. coli*, chemotaxis toward Asp, maltose and certain dipeptides is mediated by a phosphorylation cascade, terminating in CheY-P, and this effect was shown to require ACS [39]. CheY is a small cytoplasmic protein that when phosphorylated causes the flagellar motors to spin clockwise. It is phosphorylated by a kinase, CheA, that is activated in turn by transducers (chemoreceptors) and a coupling factor, CheW. Another cytoplasmic protein, CheZ, facilitates the dephosphorylation of CheY-P [40,41]. CheA and CheY are members of the homologous two-component regulatory-protein family [42,43]. Cells of *E. coli* deleted for genes that encode the transducers and all the known cytoplasmic Che proteins, except CheY, responded reversibly to the addition of acetate by spinning their flagellar motors clockwise. ACS may be involved in this process through chemical modification of CheY or a motor-switch component with which CheY interacts [44].

Tetrahymena cells are able to grow on acetate as the sole carbon source. Before it is oxidized to gain energy or used as a precursor of lipid biosynthesis, acetate must be activated to acetyl-CoA by ACS. In *Tetrahymena*, this enzyme was reported to be localized primarily on the peroxisome, at least in cells grown under conditions that favour an active glyoxylate bypass [45]. *Tetrahymena* cells undergo numerous adjustments of central metabolic pathways in response to their carbon source; in particular, the carbon flow differs in cells grown on glucose, pyruvate or acetate. It has been shown that there are two operating pools of acetyl-CoA in *Tetrahymena*. The acetyl-CoA derived most directly from acetate is associated with lipid synthesis, whereas the acetyl-CoA derived from pyruvate, and presumably localized in the mitochondria, is more closely associated with oxidation to CO₂. Glycolysis is the principal pathway for glucose metabolism [46,47]. The present work

demonstrated that the mRNA level of *TpAcs* was greatly increased by exposing the cells to low concentrations of acetate, suggesting that acetate might be a preferred substrate for *T. pyriformis* ACS and that the *TpAcs* gene can be activated, at least at the transcriptional level, by low concentrations of acetate in the medium (Figure 6). To date, all attempts to understand the possible function of ACS in *Tetrahymena* were mainly focused on carbon metabolism. We cloned the *TpAcs* cDNA during synchronous cell division by mRNA differential display. It was surprising to find that the mRNA level of *TpAcs* decreased immediately after EHT, with only approx. 10% of the original expression at 30 min after EHT; subsequently the expression level increased (Figure 5). These fluctuations indicate that changes in the *TpAcs* mRNA level are associated with the cell cycle and that *TpAcs* mRNA level may be controlled by some unknown mechanisms dependent on cell-cycle events. Furthermore, based on the possible involvement of ACS in the phospho-relay-mediated signal-transduction pathway, we propose that *TpAcs* may also, in some way, interfere with some unknown regulatory elements to control multiple cell-cycle events in *Tetrahymena*. Thus, this proposal opens a novel field for the study of cell-cycle regulation. However, further experiments are needed to prove this hypothesis.

This work was supported, in part, by the following awards: Grants-in-Aid for Scientific Research on Priority Areas (09273104, 10212204), Grants-in-Aid for Creative Basic Research (09NP0601), Grants-in-Aid for Scientific Research (B) (09480162) and (C) (10670136) from the Ministry of Education, Science, Sports and Culture of Japan. Additional funds came from the Special Co-ordination Funds for Promoting Science and Technology from the Science and Technology Agency of Japan, and the Uehara Memorial Foundation.

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Received 28 April 1999/13 July 1999; accepted 11 August 1999