Molecular cloning and cell-cycle-dependent expression of a novel NIMA (never-in-mitosis in *Aspergillus nidulans*)-related protein kinase (*TpNrK*) in *Tetrahymena* cells

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With the intention of investigating the signal-transduction pathway that mediates the cold-stress response in *Tetrahymena*, we isolated a gene that encodes a novel protein kinase of 561 amino acids, termed *Tetrahymena pyriformis* NIMA (never-in-mitosis in *Aspergillus nidulans*)-related protein kinase (*TpNrK*), by differential display from *Tetrahymena* cells exposed to temperature shift-down. *TpNrK* possesses an N-terminal protein kinase domain that is highly homologous with other NIMA-related protein kinases (Neks) involved in the control of the cell cycle. The *TpNrK* protein is 42 % identical in its catalytic domain with human Nek2, 41 % identical with mouse Nek1 and 37 % with *A. nidulans* NIMA. In addition, *TpNrK* and these NIMA-related kinases have long, basic C-terminal extensions and are therefore similar in overall structure. In order to further explore

the function of the *TpNrK* gene and the association of the cold stress with the cell cycle of *Tetrahymena*, changes of *TpNrK* mRNA were determined during the course of the synchronous cell division induced by the intermittent heat treatment. The level of *TpNrK* transcription increased immediately after the end of the heat treatment, with a peak at 30 min, and declined thereafter reaching the minimum level when nearly 80 % of the cells synchronously entered cell division (75 min after the end of heat treatment). The accumulation of *TpNrK* mRNA starting from 0 min to 30 min after the end of the heat treatment was assumed to be a prerequisite for the start of synchronous cell division. These results suggest that *TpNrK* may have a role in the cell cycle of *Tetrahymena*, and that mRNA expression, at least, is under tight cell-cycle control.

INTRODUCTION

All organisms tested so far respond to changes in their local environmental temperature by a complex signal-transduction regulatory network. This stress response is not only an interesting area for investigation but it is also an excellent model for the analysis of basic cellular processes involved in the control of gene expression [1–3]. At present, the molecular mechanisms for signal transduction by cold stress leading to modification of the expression of many genes remain to be identified.

The unicellular eukaryotic protozoan *Tetrahymena* is widely used as an *in vitro* model system to study the molecular basis of thermo-adaptive control [4–6]. It has been shown previously that *Tetrahymena* exerts striking changes in membrane lipid composition when exposed to changes in growth temperature [7–11]. The cryo-adaptive regulation of fatty acid composition can be achieved by modifying the activity of Δ^9 -desaturase (EC 1.14.99.5). Recently, Δ^9 -desaturase has been cloned and evidence has been provided to indicate that the elevated enzyme activity in chilled *Tetrahymena thermophila* cells is controlled, at least in part, at the transcriptional level [12]. However, the precise mechanisms underlying the regulation of the desaturase are still ambiguous. To investigate further the signal-transduction pathway that mediates the cold-stress response in *Tetrahymena*, we have attempted to isolate some differentially expressed genes which may be involved in this complex process. A unique PCR-based technique, termed differential display [13], is a useful approach for this purpose. One of the principal advantages of differential display is that it permits the simultaneous identi-

fication of genes that are up-regulated or down-regulated. Thus differential display has the potential to identify a spectrum of molecular factors, known and unknown, that are differentially regulated in cells under various conditions.

By using this technique, we have identified several differentially expressed transcripts during temperature shift-down in *Tetrahymena* cells, one of these is highly similar to never-in-mitosis in *Aspergillus nidulans* (NIMA)-related protein kinases (Neks), which have regulatory roles in the cell cycle in various cell types [14–24]. The integral association between cold stress and the cell cycle has incited us to clone this gene. To our knowledge, before the present work, no *nimA* functional homologue or NIMA-like activity has been found in *Tetrahymena*. Here we describe the isolation of a gene encoding a novel protein kinase that is structurally related to the *nimA* gene, termed *Tetrahymena pyriformis* NIMA-related protein kinase (*TpNrK*), and its mRNA expression during the cell cycle in *T. pyriformis*.

EXPERIMENTAL

Materials

Restriction enzymes and other nucleic-acid-modifying enzymes were obtained from Boehringer Mannheim, Toyobo and Nippon Gene. *Taq* DNA polymerase was from Takara. Radioactively labelled nucleotides, the Sequenase version 2 DNA sequencing kit and multiprime DNA labelling system were from Amersham. Arbitrary primers (AP-1–AP-10) were obtained from Gen-

Abbreviations used: NIMA, never-in-mitosis in *Aspergillus nidulans*; NrK or Nek, NIMA-related protein kinase; cdc, cell division cycle.

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Hunter. T-Vector was from Novagen. RNA size markers were from Gibco-BRL. 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

T. thermophila cells were grown in enriched proteose peptone medium (2% (w/v) proteose peptone, 0.2% (w/v) yeast extract, 0.5% (w/v) glucose) at 35 °C with shaking (85–90 strokes/min) [9] and were subjected to a temperature down-shift to 15 °C at the rate of 0.8 °C/min, as described previously [12]. For induction of the synchronous cell division, *T. pyriformis* strain W was grown in the proteose peptone medium at 26 °C and cells in the early exponential phase were subjected to the cyclic heat treatment described by Watanabe [25]. 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).

Differential display

Total RNA was extracted from *T. thermophila* by the guanidine thiocyanate method [26] and 0.2 µg RNA was reverse transcribed with SuperScript II reverse transcriptase, GT₁₅MN (V = A, C or G; N = A, C, G or T) oligo(dT) primer (1 µM) and dNTP mixture (20 µM each) at 42 °C for 60 min. The PCR, recovery and re-amplification of cDNAs obtained were performed as described previously [13,27] with slight modifications. Reverse transcriptase products from 20 ng of total RNA were amplified by PCR in 20 µl of reaction mixture containing 1 unit of *Taq* polymerase, 0.5 µM of arbitrary primers (AP-1–AP-10), 0.5 µM of the same GT₁₅MN oligo(dT) primer used for reverse transcription, dNTP (2 µM), [α -³⁵S]dCTP (10 µCi). PCR reactions were carried out for one cycle of 3 min at 94 °C, 5 min at 40 °C and 3 min at 72 °C, followed by 40 cycles of 30 s at 94 °C, 2 min at 40 °C and 1 min at 72 °C (15 min at 72 °C for the last cycle). The amplified cDNAs were separated on 6% denaturing sequence gels containing 6 M urea and then subjected to autoradiography. cDNA bands differentially amplified in cold-adapted *Tetrahymena* cells were excised from the gels and eluted by boiling. The eluted DNA was re-amplified by PCR using the same set of primers and its nucleotide sequence was determined by the dideoxy nucleotide termination method [28].

Screening of cDNA library and DNA sequencing

Amplified cDNA fragments were cloned into pT7Blue T-vector. One of the cDNA fragments, tentatively termed 4, was subjected to further analysis. *T. thermophila* and *T. pyriformis* λ gt10 cDNA libraries [29,30] were screened by plaque hybridization using a ³²P-labelled oligonucleotide probe. The cDNA insert of a positive phage clone was subcloned into pBluescriptII plasmid. Size-fractionated unidirectional deletion of the insert was performed using exonuclease III and mung bean nuclease [12].

Analysis of the putative amino acid sequences and construction of a phylogenetic tree

A homology search was performed using the BLAST algorithm [31] at the National Center for Biotechnology Information (National Library of Medicine, National Institutes of Health, Bethesda, MD, U.S.A.). Nucleotide and amino acid sequence analysis was performed with the software DNASIS. Amino acid alignment was performed using the computer program Clustalx

[32]. A phylogenetic tree was constructed by the neighbour-joining method [33] using the PHYLIP package [34].

Northern-blot analysis

Total RNAs were extracted from *T. pyriformis* and *T. thermophila* cells by the guanidine thiocyanate method [28]. Samples of total RNA (20 µg) were fractionated on a 1.0% formaldehyde denaturing agarose gel and transferred to a GeneScreen Plus membrane. Northern-blot hybridization was performed as follows: after prehybridization at 42 °C for 4 h in hybridization buffer containing 50% formamide, 5×SSPE (1×SSPE = 0.15 M NaCl/10 mM NaH₂PO₄/1 mM EDTA, pH 7.4), 0.1% (w/v) SDS, 0.1 mg/ml denatured salmon sperm DNA and 5×Denhardt's solution, filters were incubated with a ³²P-labelled probe for 16 h at 42 °C. The filters were washed twice in 2×SSC (1×SSC = 0.15 M NaCl/0.015 M sodium citrate) containing 0.1% (w/v) SDS at 45 °C for 30 min and in 0.2×SSC containing 0.1% (w/v) SDS at 55 °C for 20 min, and were autoradiographed and the density of each band was measured (Densitograph Atto, Tokyo, Japan).

RESULTS

Isolation of the NIMA-related protein kinase cDNA from *T. pyriformis*

RNA was extracted from *T. thermophila* cells which had been grown at 35 °C and, 1 h later, subjected to a shift to 15 °C at the rate of 0.8 °C/min. The RNA samples were subjected to differential display analysis using ten arbitrary primers (AP-1–AP-10) and two anchored primers (GT₁₅MG and GT₁₅MA). Approx. 1500 bands ranging from 100 to 400 bp were amplified. Although the intensities of most of the bands were not significantly different in the cells cultured at either 35 °C or 15 °C, different intensities were seen in several bands (Figure 1). Differential expression was

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

(A) Autoradiogram of typical differential display. The band (designated 4) representing differential gene expression is indicated with an arrow. (B) Northern blot of a cDNA fragment of 4, obtained by differential display. Total RNA (30 µg) was subjected to Northern-blot analysis. N, samples from cells cultured at 35 °C (normal temperature); C, samples from cells shifted down to 15 °C (cold temperature).

Figure 2 Sequence and structure of the *TpNrK* cDNA and the predicated translation product

(A) The structure of the composite cDNA is shown with the initiation and termination codons indicated. An in-frame stop codon, situated in the 5'-nontranslated region, is represented by an asterisk. Restriction endonuclease sites are also shown. The expected protein product is depicted above the composite cDNA, with the catalytic domain and C-terminal tail indicated. (B) The sequence of the composite *TpNrK* cDNA is shown together with the sequence of the expected translation product. The conserved residues of a protein kinase catalytic domain are shown in bold and with a dot. An in-frame stop codon situated in the 5'-non-translated region is underlined (line 349).

confirmed by Northern-blot analysis using these cDNA fragments as probes. A cDNA fragment (termed 4, approx. 500 bp) amplified with GT₁₅MG and arbitrary primer AP-5 recognized about 2.4 kb mRNA, which was markedly expressed in cells shifted to 15 °C (Figure 1B). Using this cDNA fragment as a probe, a *T. thermophila* *agt10* cDNA library [29] was first screened. Two positive clones were obtained from about 150000

plaques. However, these identical clones (1402 bp) did not contain full-length cDNA. Therefore the screening of a *T. pyriformis* *agt10* cDNA library [30] with the same probe was attempted. Four positive clones were isolated from about 120000 plaques and designated TpD1–TpD4. It was revealed by restriction analysis that they were identical and the insert cDNA had at least two *EcoRI* sites and a *BamHI* site. In the *T.*

Figure 3 Alignment of the amino acids comprising the catalytic domains of Neks

(Upper panel) amino acids were aligned and gaps were introduced to maximize the homology by using computer program Clustalx [32]. Upper panel: amino acid sequence comparison of *TpNrk* with mouse Nek1 (mNek1), human Nek2 (hNek2), mouse Nek2 (mNek2), *A. nidulans* NimA (AnNimA), *Neurospora crassa* Nim-1 (NcNim1), *Trypanosoma brucei* NrkA (TbNrkA), *Saccharomyces cerevisiae* Nrk (ScNrk). The approximate positions of the eleven protein kinase subdomains defined by Hanks and Quinn [36] and Hanks and Hunter [38] are shown with bold Roman numbers below the sequences. The conserved amino acid residues are boxed. (Lower panel) Schematic presentation of the primary structures of the Neks. The homologous catalytic domains are shown as black boxes (percentage identity with *TpNrk* indicated). The total number of amino acid residues (AA) and the isoelectric points (pI) of the C-terminal portions of the kinases are also shown.

pyriformis library, insert cDNAs were cloned into the *EcoRI* site of λ gt10 phages with an *EcoRI*–*Bam*HI–*Kpn*I–*Nco*I adapter. Therefore the *Kpn*I fragment of TpD1 was subcloned into the *Kpn*I site of pBluescript and the nucleotide sequence was determined (Figure 2). The cDNA contained 2408 nucleotides with a putative open-reading frame which encoded a protein of 561 amino acids with a predicted molecular mass of 64.9 kDa, a 5'-untranslated stretch of 349 nucleotides and a 3'-untranslated

region of 319 nucleotides, followed by 54 polyA residues. There was one in-frame stop codon located at nucleotide position –45, upstream of the ATG initiation codon. DNASIS analysis reveals that the coding region (1685 bp) had an A + T content of 63.6 %, while the 5' upstream (349 bp) and 3' downstream (372 bp) non-coding regions possessed an A + T content of 70.8 % and 79.9 % respectively, characteristically higher than that in the coding region. These results are in agreement with the values calculated

Figure 4 Phylogenetic tree analysis of *TpNrk*

The construction of the phylogenetic tree was based on (A) the aligned amino acid sequences of the whole protein sequences or (B) on the catalytic domain of the kinases by the neighbour-joining method [33] using the PHYLIP package [34]. The accession numbers of these kinases are: mouse Nek1 (mNek1), P51954; human Nek2 (hNek2), P51955; mouse Nek2 (mNek2), U95610; human Stk2 (hStk2), P51957; *A. nidulans* NimA, P11837; *N. crassa* Nim-1, P48479; *S. cerevisiae* Nrk, P38692; *S. cerevisiae* Kin3, S11185; *S. pombe* Nrk, Z98975; *T. brucei* NrkA, L03778.

for the overall genome of *T. thermophila* [35]. Therefore almost the full-length gene was obtained.

Properties of the predicted protein

A search of the EMBL and GenBank databases confirmed that the cloned gene encoded a novel protein and also revealed that it contained all 11 conserved subdomains of the protein kinase family and had almost all of the characteristic features of the kinases (Figure 2B) [36–38]. These included: (a) the glycine loop Gly¹⁸-X_{aa}-Gly²⁰-X_{aa}-X_{aa}-Gly²³, forming a part of the ATP binding region; (b) the catalytic loop Arg¹³⁸-Asp¹³⁹-X_{aa}-X_{aa}-X_{aa}-Asn¹⁴⁴, involved in catalysis and in guiding the peptide substrate into the proper orientation so that catalysis can occur; (c) Asp¹³⁹, Asn¹⁴⁴ and Asp¹⁵⁷, which are also identified as a sequence motif implicated in ATP binding, and (d) Glu¹⁸², Asp¹⁹⁴ and Arg²⁵⁴, which are involved in the stabilization of the protein kinase. Analysis showed that it had a high structural homology with Neks. We designated this clone as *TpNrk* (*T. pyriformis* NIMA-related protein kinase), based on its amino acid identity with the catalytic domain of Neks (Figure 3). In addition to the primary sequence similarity at the catalytic domain, *TpNrk* and the Neks are also similar in their overall structural arrangement, with their kinase domains at the extreme N-terminus, followed by a long basic C-terminal extension (Figure 2A, Figure 3B). This can be regarded as one of the structural characteristics of the Neks. The phylogenetic tree analysis also shows that *TpNrk* is a member of the Neks (Figure 4).

Cell-cycle-dependent expression of *TpNrk*

Tetrahymena cells were synchronized by the cyclic heat treatment described by Watanabe [25]. Immediately before the onset of cell division, a shortening of the length of the cells occurred. Hoechst 33258 staining revealed that nuclear divisions took place around 75 min after end of heat treatment (results not shown). After eight cycles of heat treatment, 88.3 % of the total cells exhibited synchronous cell division 75 min after the end of the heat treatment. However, the division index fell to 37.8 % and 16.6 % with two and four cycles of heat treatment respectively. RNAs were extracted from cell cultures with different division indices and analysed by RNA blots. A single band of 2.4 kb was

Figure 5 Changes in mRNA levels of *TpNrk* during the synchronous cell division induced by different cyclic heat treatments

Cells in the early exponential phase were subjected to cyclic heat treatment (26 °C for 30 min and 34 °C for 30 min/cycle). Total RNA samples (20 µg), taken at the times indicated at the end of two, four or eight cycles of heat treatment, were separated by electrophoresis on 1.0 % formaldehyde/agarose gel and transferred to nylon membranes. The membranes were hybridized with ³²P-labelled *Pst*I–*Eco*RI *TpNrk* fragment (nt 1075–1915) as a probe. The bands were detected by autoradiography. A typical autoradiogram from two different cultures is shown. The positions of RNA size markers (kb) are shown on the right (0, origin). The densities of the bands were monitored and the expression of *TpNrk* mRNA at 30 min after the end of heat treatment (EHT) is shown as fold increase relative to that at zero time. The division index at 75 min after the end of heat treatment is indicated in %.

Figure 6 Changes in the level of *TpNrk* mRNA by Northern-blot analysis during synchronous cell division

Total RNA samples (25 µg) taken at the times indicated after the end of heat treatment (EHT) were separated by electrophoresis on a 1.2 % formaldehyde/agarose gel and transferred to nylon membranes. The membranes were hybridized with a ³²P-labelled *Pst*I–*Eco*RI *TpNrk* fragment (nt 1075–1915) as a probe. Bands were detected by autoradiography. (A) Typical autoradiogram from three different cultures. The densities of the bands were monitored (B) and the results shown (fold increases above the control value) are the means ± S.D. from three different cultures.

revealed with the radioactively labelled *TpNrk* fragment (Figure 5). Quantification of *TpNrk* mRNA by densitometry showed that there were marked fluctuations in the level of these transcripts throughout the cell cycle in the well-synchronized cell culture. After eight cycles of heat treatment, the amount of *TpNrk* mRNA increased immediately after the end of heat treatment, with an almost 6-fold elevation at 30 min, and then decreased sharply before the synchronous cell division at 75 min (Figure 6). In the cell cultures exposed to four and two cycles of heat

Figure 7 mRNA expression of *TpNrk* during heat treatment

(A) Cells grown at 26 °C were exposed to cyclic heat treatment and sustained heat treatment (shown by the bars beneath the blot, the arrowhead shows the start point at which the cells were exposed to heat treatment) and were harvested at the indicated times and temperatures. (B) Cells, after 8 cycles of heat treatment, in synchronous cell division were harvested at the indicated times after the end of heat treatment (EHT). The mRNA levels of *TpNrk* were determined by Northern-blot analysis as described in the legend to Figure 5. A typical autoradiogram from two different cultures is shown.

treatment, the fluctuation in *TpNrk* transcription during the cell cycle was almost identical with that induced by eight cycles of heat treatment, but the levels of expressed mRNAs were much lower, with an approx. 4- and 2-fold increase at 30 min after the end of heat treatment respectively (Figure 5). Thus the expression levels of *TpNrk* at 30 min after the end of heat treatment were coincident with the division indices. These findings led us to consider that the changes in the mRNA level were related to the cell cycle.

To further verify the cell-cycle-associated changes in *TpNrk* mRNA levels, the mRNA expression of *TpNrk* was investigated during the heat treatment. The results indicated that when cells grown at a permissive temperature (26 °C) were shifted to a restrictive temperature (34 °C), the expression of *TpNrk* was down-regulated to about 50 % of the steady-state level expressed at 26 °C (Figure 7). Upon shift to, and incubation at, 26 °C for 30 min, the expression of *TpNrk* was only slightly increased. These observations indicated that the heat treatment itself was not a cause of the augmented expression of *TpNrk* before mitosis.

DISCUSSION

There is mounting evidence that reversible protein phosphorylation plays a key role in controlling progression through the cell cycle in all eukaryotes. The universal regulator of the cell cycle in mitotic cells is the serine/threonine protein kinase p34^{cdc2} and structurally-related cyclin-dependent kinases, which function with their regulatory partner, cyclin, to control distinct transitions within the cell cycle, particularly entry into mitosis and the initiation of DNA synthesis [39,40]. Although the importance of cyclin-dependent-kinase-cyclin complexes in cell-cycle progression is well established, these kinases are unlikely to function as the sole regulator of the cell cycle. There is some evidence that other regulatory pathways may co-operate with cyclin-dependent-kinase-cyclin complexes in controlling cell-cycle progression [41,42]. In the filamentous fungus *A. nidulans*, a serine/threonine-specific protein kinase, NIMA, has been implicated in controlling entry into mitosis, although it is distinct from cell division cycle (*cdc*)2 kinase, structurally as well as biochemically [43,44]. A temperature-sensitive *nimA* allele arrests cells in G₂ at the non-permissive temperature [45], whereas overexpression of the NIMA protein kinase drives cells into mitosis at any stage of the cell cycle. Most interestingly, cells expressing inactive NIMA are arrested in G₂ [46], despite the fact that they exhibit active p34^{cdc2}, and cells expressing inactive

p34^{cdc2} are arrested in G₂ with active NIMA [43]. These data indicate that p34^{cdc2} and NIMA may be activated independently, and that the activation of both kinases is required for entry into mitosis. Evidence recently accumulated suggests that NIMA is activated and phosphorylated by p34^{cdc2}-cyclin B during mitotic initiation, indicating that NIMA may function either downstream or in parallel to p34^{cdc2} in order to promote mitosis [47]. Several genes that encode protein kinases which are significantly similar to *A. nidulans* NIMA, termed *Nek1*, *Nek2*, *Nek3* and *Stk2*, have been cloned recently from mammalian species [17,20,21,24]. Two *nimA*-like genes have been isolated from yeast (*kin3* and *nrk*) [15,22] and two related genes have been isolated from *Trypanosoma* [19], but the role of NIMA-related kinases in cell cycle regulation remains to be established.

In the present study, we have successfully isolated a gene encoding a novel protein kinase, *TpNrk*, from the unicellular eukaryotic protozoan *T. pyriformis*. The partial fragment of its counterpart was initially isolated by differential display from *T. thermophila* cells, exposed to temperature shift-down. Database analysis revealed that it belongs to the NIMA-related protein kinases family. Thus we have called this gene *TpNrk*. The C-termini of the NIMA-related kinases known to date show little similarity either in size or amino acid sequence, but it is noteworthy that members of this family are highly basic (Figure 3B). Over the catalytic domains, *TpNrk* shares, at the amino acid level, the highest identity (42 %) with human Nek2 [20], 41 % with murine Nek1 [17] and 37 % with NIMA [14]. The sequence shown in the present work possesses the characteristics of this family in these two subdomains (Figure 3A). Phylogenetic tree analysis also reveals that *TpNrk* is a member of the NIMA-related kinase family (Figure 4). At present, the relationship between *A. nidulans* NIMA and mammalian Nek1, Nek2 and Nek3 is primarily based on the structural similarities. It remains uncertain to what extent these mammalian kinases are functionally related to NIMA. However, these and/or related kinases have roles in the progression of the eukaryotic cell cycle, because expression of the active *A. nidulans* NIMA induces, but the dominant-negative NIMA blocks, germinal vesicle breakdown in *Xenopus* oocytes and premature mitotic events in HeLa cells [41].

In ciliates, cell-cycle progression has been thought to involve *cdc2* kinase homologue(s) and a cyclin-like function has been suggested in *Tetrahymena* cells, although it has not yet been identified [48]. Until the present work, no *nimA* homologue and NIMA-like activity have been identified in *Tetrahymena*. In order to explore the function of the *TpNrk* gene in *Tetrahymena*, changes in *TpNrk* mRNA were investigated in synchronous cell divisions in *T. pyriformis*, induced by different cycles of heat treatment (Figure 5). Following the eighth cycle of heat treatment, 83.3 % of cells synchronously entered cell division 75 min after the end of heat treatment. The mRNA level of *TpNrk* was increased immediately after the end of heat treatment, with an almost 6-fold elevation at 30 min, and thereafter declined to the lowest level 75 min after the end of heat treatment. In the case of synchronous cell divisions induced by two and four cycles of heat treatment, the division index fell to 27.8 % and 16.6 % respectively. The overall fluctuations in *TpNrk* mRNA before mitosis were similar to those induced by eight cycles of heat treatment, but the levels of *TpNrk* transcription was much lower, with only a 4- or 2-fold elevation 30 min after the end of heat treatment. In the three types of synchronous cell culture, the expression levels of *TpNrk* transcripts 30 min after the end of heat treatment correlated with the division indices. These results indicated that the changes in the *TpNrk* mRNA level are associated with the cell cycle. To ascertain the relationship between the *TpNrk* expression and the cell cycle of *T. pyriformis*,

we examined the *TpNrK* mRNA expression during the heat treatment (Figure 7). When cells grown at 26 °C were exposed to 34 °C, the expression of *TpNrK* was reduced to about half of the level expressed at 26 °C and was only slightly increased upon shift to and incubation at 26 °C for 30 min. Thus we may conclude that the increased expression of *TpNrK* before mitosis was not due to the heat treatment itself. Taken together, the above results strongly suggest that the elevated *TpNrK* mRNA level at 30 min after the end of heat treatment is a prerequisite for the start of cell division and that the level of *TpNrK* mRNA may be controlled by some unknown mechanism, depending on the cell cycle events. In *A. nidulans* synchronously dividing cells, the level of the NIMA is elevated as cells enter mitosis and drops sharply as cells progress through mitosis and, in cells blocked in S phase, there is a very low level of *nimA* mRNA, whereas, in cells blocked in mitosis the elevated level of the *nimA* transcript is maintained. These data demonstrated that *nimA* is required for entry into mitosis because the transcript is cyclically expressed [49]. The role of Neks in cell cycle regulation is yet to be defined, but recent data showing that mouse Nek1 is highly expressed in germ-line cells suggests a role in meiosis [17]. In addition, the level of human Nek2 is regulated through the cell cycle, reaching a maximum during G₂ phase, which indicates that Nek2 may also play a cell-cycle-specific role in humans [20,50]. A recent report suggests that Nek2 is located at the centrosome and may regulate its separation during mitosis [51]. The cell-cycle-dependent changes in the *TpNrK* mRNA level are similar to those observed in NIMA during the cell cycle of *A. nidulans*. From these results, we conclude that *TpNrK* may also play some part in the cell cycle of *Tetrahymena*, and, at least, that mRNA expression is under tight cell-cycle control.

Our initial idea was to isolate the differentially expressed genes during cold stress in *Tetrahymena* cells so that some clues to the molecular mechanisms for the stress signaling pathway might be found. The isolation of a novel NIMA-related kinase (*TpNrK*), which may be involved in regulation of the cell cycle of *Tetrahymena*, was of great interest. However, the potential function of this gene in *Tetrahymena* and the signal cascade via stress stimuli to the cell cycle still remain to be explored.

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