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Cell Cycle- and Terminal Differentiation-Associated Regulation of the Mouse mRNA Encoding a Conserved Mitotic Protein Kinase

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We determined the nucleotide sequence of a mouse and a human cDNA, which we designate STPK13, that encodes an apparent protein kinase related to that encoded by the *Drosophila melanogaster polo* gene and the *Saccharomyces cerevisiae CDC5* gene. The *polo* and *CDC5* gene products are required for normal mitosis. The STPK13 mRNA is regulated during terminal erythrodifferentiation and during the cell cycle. Within the precommitment period of murine erythroleukemia cell terminal differentiation, most of the poly(A) tail is lost from the STPK13 mRNA, but the body of the mRNA remains unchanged in abundance; this poly(A) loss does not occur in mutant erythroleukemia cells that fail to commit to terminal differentiation. During the cell cycle, the abundance of the body of the STPK13 mRNA fluctuates. The mRNA is present in growing but not in nongrowing cells. It reaches a maximum abundance during G_2/M phase, is absent or present at only low levels during G_1 phase, and begins to reaccumulate at approximately the middle of S phase. The cell cycle-associated accumulation and loss of the STPK13 mRNA could cause a similar fluctuation in abundance of its encoded protein kinase, thereby providing a maximum amount during M phase, when the kinase is thought to function, and little or none at other times of the cell cycle. Posttranscriptional regulation must be responsible for the cell cycle-associated fluctuations because transcription rates are relatively constant during different times of the cell cycle when there are large differences in mRNA abundance.

Protein kinases are pivotal in a variety of cellular processes, including the orderly progression of events by which cells grow, replicate DNA, divide, and undergo various differentiation processes (14, 18, 21). Many homologous protein kinases are present in diverse eukaryotic species (9, 12, 14, 21), suggesting that at least some of the cellular processes in which they participate have been evolutionarily conserved. During a search for genes that function to accomplish commitment to terminal erythrodifferentiation, we were prompted to identify mRNAs for protein kinases that might participate in the processes of commitment or in the postcommitment events that allow the expression of differentiation-specific gene products. It was previously demonstrated that inhibitors of protein tyrosine kinases cause the accumulation of globin mRNAs in otherwise uninduced murine erythroleukemia cells (MELC) (26). We therefore searched for poly(A)-terminated mRNAs that encode putative protein kinases that might be down regulated during the precommitment stage of induced terminal differentiation of cultured MELC. We first used a polymerase chain reaction method (28) to identify putative protein kinase mRNAs in uninduced MELC and subsequently examined them in MELC induced to undergo terminal erythrodifferentiation to identify those that decline during the precommitment stage. We found an mRNA that fulfills these criteria. Sequence comparisons revealed that the protein kinase encoded by this mRNA is extensively homologous to that encoded by the previously characterized *Drosophila polo* gene and the *Saccharomyces cerevisiae CDC5* gene, which are required for proper mitosis (5, 10, 17, 24). Further analysis revealed the mRNA to be present in a variety of growing cells and absent from nongrowing cells. It periodically fluctuates in abundance during the cell cycle, with a maximum at the G_2/M phase, when its encoded protein kinase is thought to function, and little or none detectable during the G_0 and G_1 phases. Nuclear runoff experiments demonstrated that there is little difference in transcription rates of STPK13 RNA at different times during the cell cycle when there are large differences in STPK13 mRNA abundance, suggesting that posttranscriptional regulation is responsible for the cell cycle-associated fluctuations in STPK13 mRNA abundance.

MATERIALS AND METHODS

Cell growth and cell cycle synchronization. MELC (line DS19) were grown in Iscove's medium supplemented with 15% fetal bovine serum and were induced to undergo terminal differentiation by addition of 5 mM (final concentration) *N,N'*-hexamethylenebisacetamide (HMBA) to the growth medium. NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Growth arrest was achieved by reducing the serum concentration in the growth medium to 0.5% for 48 h. Growth-arrested cells were stimulated to resume growth by adding fresh growth medium containing 15% fetal bovine serum. To obtain mitotic cells, growth-arrested NIH 3T3 cells were stimulated with medium containing 15% fetal bovine serum for 6 h, at which time aphidicolin (5 μg/ml, final concentration) was added to arrest the cells at the beginning of the S phase (20) to maintain cell cycle synchrony. Twelve hours later, the aphidicolin-containing growth medium was removed and fresh medium without aphidicolin was added. Seven hours later, when mitotic cells first began to appear, nocodazole (0.4 μg/ml, final concentration) was added to the growth medium to halt mitosis (19).

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Seven hours after nocodazole addition, nonadherent, mitotic cells were collected.

Measurement of DNA content and DNA synthesis. Cellular DNA content was determined by staining cells with propidium iodide and measuring fluorescence in a Becton Dickinson FACScan. For each cell population, 10,000 cells were analyzed, and the proportions in G_1, G_2/M, and S phases were estimated by using Modfit cell cycle analysis software (Verity Software, Topsham, Maine). DNA synthesis was assayed by measuring the amount of acid-insoluble [*methyl*-^{3}H]thymidine incorporated by cultures of cells during a standard time period.

RNA isolation and analysis. Total cell RNA was isolated by the acid guanidine method of Chomczynski and Sacchi (3). Poly(A)-terminated RNA was isolated from total cytoplasmic RNA by oligo(dT)-cellulose chromatography (1). Northern (RNA) blot analyses were performed as described by Thomas (25). RNA was subjected to electrophoresis in 0.8% agarose-formaldehyde gels and transferred to Duralon membranes (Stratagene Cloning Systems). The membranes were treated with UV light in a UV Stratalinker (Stratagene Cloning Systems), hybridized with radiolabeled probe DNA in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 42°C, washed at 65°C in 0.2× SSC, and exposed to X-ray film.

cDNA synthesis and cloning. Single-stranded cDNA was synthesized from poly(A)-terminated RNA by avian myeloblastosis virus reverse transcriptase as described by Gubler and Hoffman (7). Polymerase chain reaction amplifications to obtain potential protein kinase-encoding cDNAs were performed with single-stranded cDNA as the template and degenerate primer oligonucleotides specific for a conserved region of the enzymatic domain of protein kinases as described by Wilks (28). Double-stranded cDNA was synthesized from uninduced MELC poly(A)-terminated RNA by the method of Gubler and Hoffman (7), ligated to *Eco*RI linkers, and cloned into the *Eco*RI restriction site of the Bluescript plasmid. A mouse testis cDNA library was obtained from Clontech Laboratories, and a human teratocarcinoma cell cDNA library was constructed by Skowronski and Singer (23).

Nuclear RNA synthesis and analysis. RNA synthesis in isolated nuclei was accomplished by the method of Greenberg and Ziff (6) and hybridized to slot blots of plasmid DNAs in 50% formamide–5× SSC at 42°C. Following hybridization, the filters were washed in 2× SSC at 65°C, treated with 10 μg of RNase A per ml in 2× SSC at 37°C, and subsequently washed in 2× SSC at 37°C.

Sequence analysis. DNA and deduced protein sequences were compared with the GenBank data base by using FASTA (22) and other programs supplied as part of the Genetics Computer Group package, version 7 (April 1991).

Nucleotide sequence accession number. The nucleotide sequences shown in Fig. 1 have been deposited in GenBank under accession numbers L19558 (mouse sequence) and L19559 (human sequence).

RESULTS

Identification of a protein kinase mRNA regulated during MELC terminal differentiation. To search for protein kinases that might be down regulated in the process of commitment to terminal differentiation or in the expression of differentiation functions, we first sought to obtain cDNA clones for such putative enzymes in uninduced MELC. Degenerate oligonucleotides corresponding to a region of conserved sequence encoding the enzymatic domain of protein tyrosine kinases (see Materials and Methods) were used as primers in a polymerase chain reaction containing cDNA synthesized from uninduced MELC poly(A)-terminated mRNA as the template. Products of expected length were ligated into a plasmid vector, and the nucleotide sequences of approximately 70 different clones were determined. More than 90% of the clones corresponded in sequence to a previously described, putative protein kinase, FD17, from the mouse (28). The remaining clones, which we term STPK13, were all identical to one another.

Preliminary Northern blot analysis of poly(A)-terminated RNA showed that the abundance of the STPK13 mRNA decreased approximately sevenfold in MELC induced for 24 h compared with its abundance in uninduced MELC, while the FD17 mRNA showed a slight increase in abundance (data not shown). The FD17 mRNA was not studied further. The dramatic decrease of the STPK13 mRNA abundance in the 24-h-induced MELC suggested that a reduction of the cellular content of this mRNA might be an event of the commitment process or the postcommitment processes required for the expression of differentiation-specific functions.

Cloning and sequencing full-length coding regions of the mouse and human STPK13 mRNAs. Clones encoding the full-length mouse STPK13 protein were isolated from cDNA libraries prepared from MELC and mouse testis; for sequence comparisons, a homologous human clone was isolated from a cDNA library prepared from human teratocarcinoma cells, and an overlapping genomic clone was isolated from a library prepared from mouse liver nuclear DNA. The sequences determined from the mouse and human cDNA clones are aligned in Fig. 1. The mouse sequence, which was determined from two overlapping clones, is 2,190 nucleotides long, including 15 AMP residues of the mRNA 3′-terminal poly(A). Attempts to locate the true 5′ end of the mouse STPK13 mRNA by primer extension either with conventional reverse transcriptase at 42°C or with the reverse transcriptase activity of thermal stable polymerases at a variety of temperatures above 60°C have consistently failed to reveal extensions templated by poly(A)-terminated MELC cytoplasmic RNA upstream of nucleotide 42, although extension products ending at nucleotide 42 were observed several times. Nonetheless, it is clear that the sequence 5′ of nucleotide 42 shown in Fig. 1 is part of the STPK13 mRNA because it is colinear with genomic DNA sequence in the 5′ direction as far as residue 9, where the genomic DNA contains an intron at least 2 kb long. The true 5′ end of the mRNA remains unknown. We have not attempted to isolate clones that contain the true 5′ end of the human mRNA. Northern blot analysis of the STPK13 poly(A)-terminated mRNA (Fig. 2) indicates that it is between 2.2 and 2.3 kb long, suggesting that the location of its 5′ end must not be more than 50 to 100 nucleotides upstream of the 5′ end of the cDNA sequence reported in Fig. 1. The human nucleotide sequence, determined from two overlapping cDNA clones, is 83% identical to the mouse sequence overall and 88% identical in the protein-coding region. The longest open translational reading frame in both the human and mouse sequences begins with a methionine codon within the context of an optimal translation initiation consensus sequence (16) and encodes a protein of 603 amino acids. Coupled, cell-free transcription-translation of our longest mouse cDNA clone, which extends from nucleotide 39 to the 3′-most nucleotide of the sequence shown in Fig. 1, and

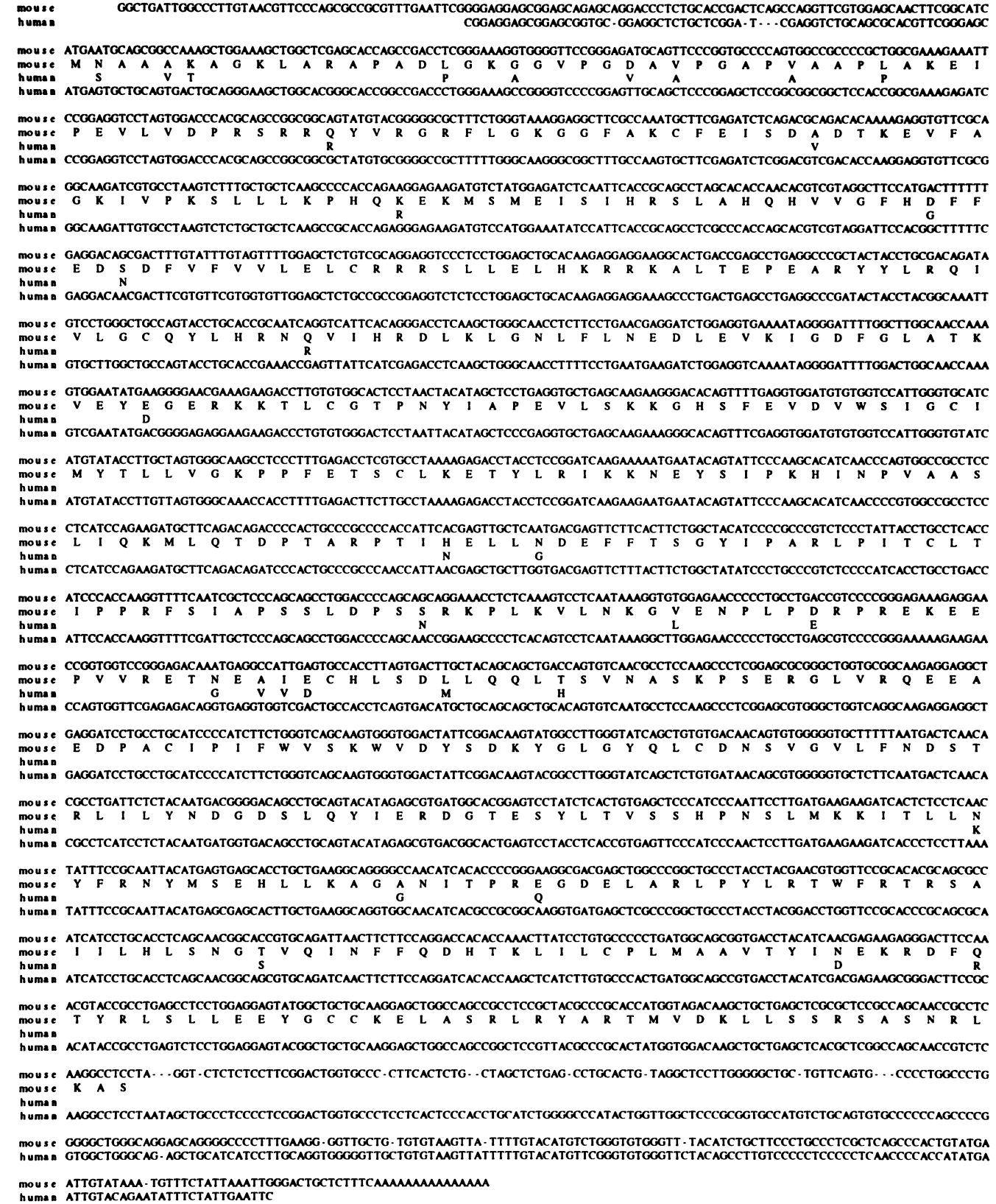

FIG. 1. Comparison of mouse and human STPK13 cDNA nucleotide and derived protein sequences. The nucleotide and derived protein sequences of the mouse and human STPK13 cDNAs are shown aligned. Dashes were introduced to allow optimal alignment. A blank space at any position in the derived human protein sequence indicates the same amino acid as in the mouse-derived protein sequence at that position.

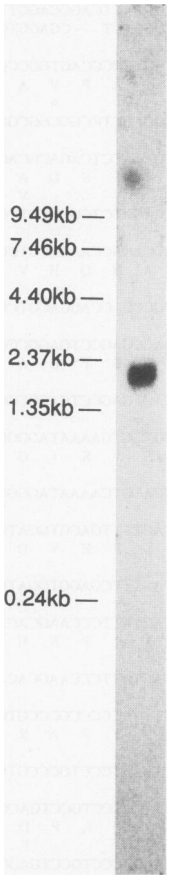

FIG. 2. Northern blot analysis of MELC poly(A)-terminated STPK13 RNA. Five micrograms of uninduced MELC poly(A)-terminated RNA was subjected to electrophoresis in a 0.8% agarose–formaldehyde gel. An adjacent lane was loaded with RNA size markers (0.24- to 0.95-kb RNA ladder; Gibco BRL). The RNA was transferred to a Duralon membrane and probed with radiolabeled mouse STPK13 cDNA sequences as described in Materials and Methods. Positions of marker RNAs are indicated.

which contains the entire protein-coding sequence, produces a protein product of the expected molecular weight, as do COS cells transfected with an expression plasmid containing the same cDNA (data not shown). The predicted proteins from the two species are 94% identical and, if conservative amino acid changes are included, 97% similar to one another. The predicted amino acid sequences of the putative N-terminal catalytic domains of the mouse and human proteins are, respectively, 66 and 65% identical to the putative N-terminal catalytic domain of the *Drosophila polo* protein kinase (17), and both the mouse and human proteins are 81% similar to the *polo* protein in this region. The remaining C-terminal regions of the mouse and human proteins are, respectively, 44 and 43% identical, and both are 65% similar, to the corresponding C-terminal region of the *polo* protein kinase. Additionally, the predicted amino acid sequences of the putative catalytic domains of both the mouse and human proteins are 51% identical and, respectively, 70 and 71% similar to the predicted protein sequence of the putative N-terminal catalytic domain of the *S. cerevisiae* CDC5 protein (15); in the remaining C-terminal region, the mouse and human proteins are, respectively, 30 and 27% identical and 56 and 52% similar to the corresponding region of the CDC5 protein. These observations suggest we have cloned cDNAs encoding the mouse and human homologs of the *Drosophila polo* and *S. cerevisiae CDC5* protein kinases.

While this report was in preparation, Clay et al. (4) reported the sequence of a mouse cDNA related to the *polo* gene. Although the sequence reported by Clay et al. (4) and that described here are extensively similar, they are not identical. There are several single nucleotide differences in the protein-coding region, which result in different predicted amino acids near the N terminus. At each position of sequence difference, our mouse cDNA and genomic sequences are identical. The 5′ nontranslated sequence reported by Clay et al. (4) is substantially longer than ours and would result in a putative mRNA larger than we observe by Northern blot analysis (Fig. 2). We are able to explain the differences between our sequence and that reported by Clay et al. (4) because we obtained a second mouse genomic clone, which appears to be a processed pseudogene since it contains no introns. It contains a region of sequence that is 96% identical to the sequence downstream of nucleotide 64 of our mouse cDNA sequence, but upstream of nucleotide 64, our cDNA and pseudogene sequences diverge completely. The pseudogene must, therefore, be truncated 5′ of nucleotide 64. Likewise, upstream of nucleotide 64, our cDNA sequence diverges completely from the 421 nucleotides of sequence reported by Clay et al. in this region. These 421 nucleotides are, however, greater than 99% identical to the genomic sequence immediately upstream of the 5′-truncated pseudogene. The sequence reported by Clay et al. (4) was obtained from three overlapping clones. Their 5′-most clone, which they called clone NE4, must be a genomic DNA clone contaminating their cDNA library, and their reported sequence must be a composite of bona fide cDNA sequence, pseudogene sequence, and genomic sequence 5′ of the truncated pseudogene.

Loss of the STPK13 mRNA poly(A) is a precommitment event. The longest mouse cDNA clone was used as a probe to examine the behavior of the STPK13 mRNA following addition of inducer to wild-type MELC and to mutant MELC that fail to commit to terminal differentiation in response to conventional inducers. These mutant MELC were isolated because, unlike wild-type MELC, they fail to cease cell division in the presence of inducers of terminal differentiation (27). Additionally, they fail to express differentiation-specific functions. Figure 3A (WT) shows that the abundance of STPK13 poly(A)-terminated cytoplasmic RNA declined dramatically in the wild-type MELC induced for only 12 h and then remained at approximately the same lower level throughout the duration of the induction time, up to 48 h. Densitometer scans of the radioautographs from two experiments showed an approximately sixfold reduction in abundance of the RNA at 12, 24, and 48 h of induction. In contrast, the abundance of the mRNA for the small ribosomal subunit protein S12, used as a control and shown in Fig. 3A, remained constant, as did 11 other mRNAs that we also investigated during the course of this work. α-Globin mRNA abundance increased during the induction time, as expected of MELC committed to the terminal erythrodifferentiation program. Unlike the large decline in STPK13 poly(A)-terminated mRNA abundance that occurred when wild-type MELC were treated with HMBA, only a slight reduction in STPK13 poly(A)-terminated mRNA abundance was observed when the mutant MELC were treated with inducer (Fig. 3A, MUT). Like the abundance of S12 mRNA in wild-type MELC treated with inducer, S12 mRNA abundance in the mutant MELC remained constant, and since

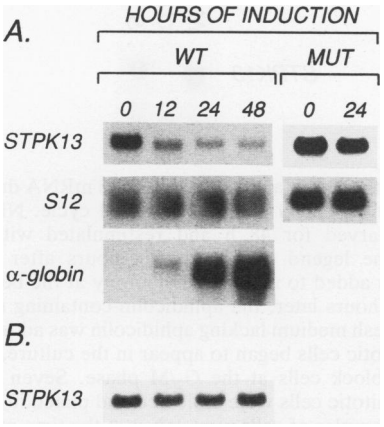

FIG. 3. Northern blot analysis of poly(A)-terminated and total cytoplasmic STPK13 RNA from wild-type and mutant MELC during induction of terminal differentiation. Cytoplasmic RNA was isolated from wild-type (WT) and inducer-refractory, mutant (MUT) MELC at the indicated times after addition of 5 mM HMBA to the culture medium. Poly(A)-terminated RNA was isolated by chromatography on oligo(dT)-cellulose. Two micrograms of poly(A)-terminated (A) or 20 μg of total cytoplasmic (B) RNA was subjected to Northern blot analysis as described in Materials and Methods. The Northern membranes were first hybridized with radiolabeled mouse STPK13 cDNA sequences and subsequently rehybridized with radiolabeled cDNA sequences encoding the mouse S12 ribosomal protein (panel A only) and mouse α-globin (panel A, WT only).

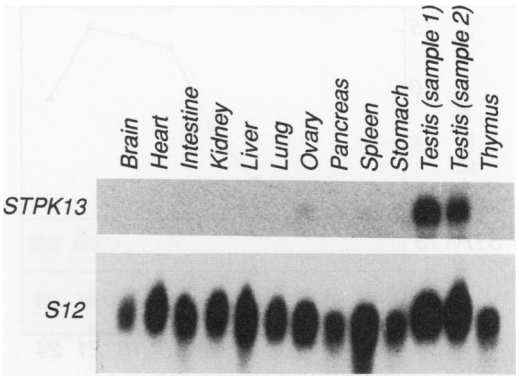

FIG. 4. Northern blot analysis of STPK13 mRNA in various mouse tissues. Twenty micrograms of total cell RNA from each of the indicated tissues from an 8-week-old Swiss Webster mouse was subjected to Northern blot analysis as described in Materials and Methods. The Northern membrane was first hybridized with radiolabeled mouse STPK13 cDNA sequences and subsequently rehybridized with radiolabeled cDNA sequence encoding the mouse S12 ribosomal protein.

globin mRNAs do not accumulate in the mutant cells in response to inducer (27), results from a globin probe are not shown. The sixfold decline in STPK13 poly(A)-terminated mRNA abundance observed in the induced wild-type MELC, but not in the mutant MELC that fail to commit to terminal differentiation, must be associated with the terminal differentiation process and not due merely to an irrelevant side effect of inducer in the culture medium.

Wild-type MELC fail to commit to terminal erythrodifferentiation if exposed to inducer for less than 12 to 16 h, but once this precommitment phase is passed, the MELC become committed to proceed through terminal differentiation even if subsequently removed from inducer (8). Differentiation-specific functions are not expressed until after commitment has occurred. The decline in STPK13 poly(A)-terminated mRNA abundance during the precommitment period suggests it is an early event of differentiation, possibly a requirement for commitment to occur.

The decline in oligo(dT)-selected STPK13 mRNA following MELC induction was not reproduced when the total cytoplasmic RNA population was analyzed. Rather, total cytoplasmic STPK13 mRNA remained at a constant level of abundance 0, 12, 24, and 48 h after addition of inducer to a culture of MELC (Fig. 3B). The decline in abundance of oligo(dT)-selected STPK13 mRNA following MELC induction must, therefore, reflect a decline in the proportion of STPK13 mRNA molecules with poly(A) tails of sufficient length to be retained on the oligo(dT) column under standard conditions (see Materials and Methods) and not due to a decline in the total abundance of this RNA. Upon MELC induction, either (i) poly(A)-terminated STPK13 mRNAs lose all or most of their poly(A) tail or else (ii) newly synthesized STPK13 mRNA molecules are not polyadenylated at all or the added poly(A) is unusually short and incapable of making stable associations with the oligo(dT).

The STPK13 mRNA is restricted to proliferating cells. Preliminary Northern blot analysis (data not shown) indicated that the STPK13 mRNA is expressed in several nonerythroid cultured cell lines, including MPC11 (mouse plasmacytoma), L cells (mouse connective tissue fibroblast), NIH 3T3 (mouse embryonic fibroblast; see below), CHO (Chinese hamster ovary), and HeLa (human cervical carcinoma). To examine a greater variety of tissue types, we surveyed RNA from 12 different mouse tissues for hybridization to the STPK13 probe. Of the tissues examined, only testis RNA showed a strong hybridization signal (Fig. 4). Ovary and spleen RNAs showed weak hybridization signals, as did intestine, liver, pancreas, and thymus RNAs, but signals from the latter four could be observed only after overexposure of the X-ray film. RNA from brain, heart, lung, kidney, and stomach showed no hybridization signal, even after excessive overexposure of the film. All of these tissues except testis either lack or have a relatively low proportion of dividing cells; testis contains a large proportion of dividing cells. These results, in concert with the observation that several lines of actively growing cultured cells contain readily detectable STPK13 mRNA, suggest that the STPK13 mRNA is present in dividing cells and absent from nondividing cells, a hypothesis that was tested directly with cultured NIH 3T3 cells.

The abundance of the STPK13 mRNA is regulated during the cell cycle. Serum starvation causes NIH 3T3 cells to withdraw from the cell cycle and growth arrest in G_0; subsequent readdition of serum to the growth medium allows the cells to reenter the cell cycle synchronously. NIH 3T3 cells were serum starved for 48 h and then restimulated with serum. Samples of cells were taken for RNA isolation and measurement of [*methyl*-^{3}H]thymidine incorporation at the time of serum addition and every 3 h thereafter for a total of 24 h. Figure 5 shows that growth-arrested cells did not contain an easily detectable amount of STPK13 mRNA in the total cellular RNA population. After serum was replenished in the growth medium, the mRNA reappeared at approximately the middle of the S phase of the cell cycle and accumulated to maximal levels at the end of S phase. A densitometer scan of the X-ray film showed a difference of at least 15-fold in the abundance of the STPK13 mRNA between its lowest level at G_0 and its highest level at the end of

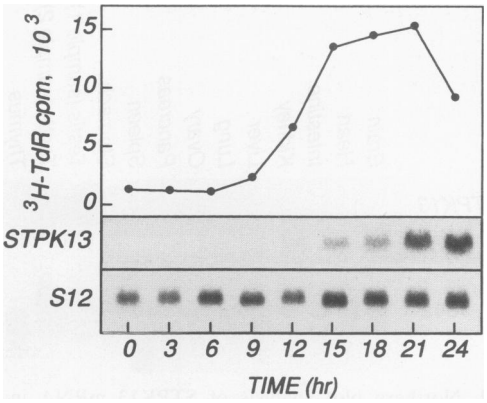

FIG. 5. Northern blot analysis of STPK13 mRNA following stimulation of serum-starved NIH 3T3 cells. NIH 3T3 cells were growth arrested by being plated in medium containing 0.5% fetal bovine serum for 48 h, at which time the cells were stimulated by being placed in fresh medium containing 15% fetal bovine serum. Total cell RNA was extracted from samples of cells at the time of the medium change (time 0) and every 3 h thereafter for a total of 24 h. Twenty micrograms of RNA from each sample was subjected to Northern blot analysis as described in Materials and Methods. The Northern membrane was first hybridized with radiolabeled mouse STPK13 cDNA sequences and subsequently rehybridized with radiolabeled cDNA sequence encoding the mouse S12 ribosomal protein.

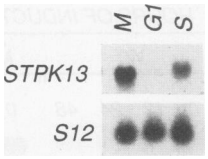

FIG. 6. Northern blot analysis of STPK13 mRNA during mitosis and subsequent G_1 and S phases of the cell cycle. NIH 3T3 cells were serum starved for 48 h and restimulated with serum as described in the legend to Fig. 5. Six hours after stimulation, aphidicolin was added to maintain synchrony at the beginning of S phase. Twelve hours later, the aphidicolin-containing medium was removed and fresh medium lacking aphidicolin was added. Six hours later, when mitotic cells began to appear in the culture, nocodazole was added to block cells at the G_2/M phase. Seven hours later, nonadherent, mitotic cells were collected and plated in nocodazole-free medium. Samples of cells were taken at the time of mitotic cell collection (M) and at G_1 phase (G1) and S phase (S), 12 and 18 h, respectively, after plating in nocodazole-free medium. Total cell RNA was extracted from each cell sample, and 20 μg of each RNA was subjected to Northern blot analysis as described in Materials and Methods. The Northern membrane was first hybridized with radiolabeled mouse STPK13 cDNA sequences and subsequently rehybridized with radiolabeled cDNA sequence encoding the mouse S12 ribosomal protein. Flow cytometry was used to measure the DNA content of each cell sample. The proportion of total cells in each stage of the cell cycle is given in the text.

S phase, although the lower measurement is subject to greater inaccuracy due to an extremely weak signal. Two interpretations are consistent with this observation. The absence of the STPK13 mRNA in serum-starved cells might be a property of cells that have withdrawn from the cell cycle, and its failure to appear in the G_1 and early S phases could merely reflect the time required, after cells reenter the cell cycle, for it to accumulate to levels high enough to be readily detected. Alternatively, the STPK13 mRNA may never accumulate in the G_1 or early S phase of the cell cycle, even in continuously growing cells. The experiment of Fig. 5, if extended for longer times, would not allow an unambiguous distinction between these possibilities because cells stimulated by serum addition do not enter the second cell cycle with a high enough degree of synchrony. We therefore used a different synchronization protocol to examine STPK13 mRNA abundance during the first M phase and the second G_1 and S phases following reentry of growth-arrested cells into the cell cycle.

NIH 3T3 cells were growth arrested by serum starvation for 48 h. Serum was replenished in the growth medium, and nocodazole was added before the first M phase occurred, to block mitosis (see Materials and Methods). Nonadherent, mitotic cells were collected from the culture and plated in fresh medium without nocodazole. Samples of cells were taken at the time of release from the nocodazole block, at late G_1 phase and at S phase for assay of STPK13 mRNA content by Northern blot analysis, and for assay of DNA content by flow cytometry. Figure 6 shows that the STPK13 mRNA was present in the M-phase (nocodazole-blocked) cells, was absent from the G_1-phase cells, and reappeared in the S-phase cells. Flow cytometry showed that 85% of the cells in the M-phase sample had 4n DNA content and 77% of the cells in the G_1-phase sample had 2n DNA content. DNA content in the S-phase sample was distributed between 2n and 4n. We conclude from this experiment and that of Fig. 5 that STPK13 mRNA abundance is regulated during the cell cycle. It reaches a maximum during late G_2/M phase, is absent or present at a very low level during the G_1 phase, and begins to accumulate during the S phase.

The reappearance of the STPK13 mRNA during S phase of the cell cycle suggested that successful completion of the S phase might be required for the mRNA to accumulate, and accordingly we investigated this possibility. Five flasks of cells were serum starved, and total cellular RNA was isolated from one of them. Serum was replenished in the growth medium of the remaining four flasks. Six hours later, before DNA synthesis began, cells were harvested for RNA isolation from one of the flasks and aphidicolin was added to two flasks to inhibit DNA synthesis. Twenty-four hours later, RNA was isolated from the cells of one of the aphidicolin-treated flasks and from the cells of the remaining untreated flask. At this time, aphidicolin was removed from the remaining flask, and 24 h later, RNA was isolated from the cells in it. The RNAs were assayed for STPK13 mRNA abundance by Northern blot analyses (Fig. 7). Parallel flasks of cells were used to monitor [*methyl*-^{3}H]thymidine incorporation into DNA at each of these times (Fig. 7, legend). At the time of serum replenishment (G_0) and 6 h later (G_1), the STPK13 mRNA was not readily detected, in agreement with the experiments of Fig. 5 and 6. Aphidicolin treatment for 24 h, which completely prevented [*methyl*-^{3}H]thymidine incorporation, resulted in a reduced, but not in a lack of, accumulation of the mRNA. Therefore, although the STPK13 mRNA begins to accumulate midway through the S phase of the cell cycle, its accumulation is apparently not obligatorily coupled to successful DNA synthesis.

Transcriptional rates of STPK13 RNA do not account for cell cycle-associated mRNA fluctuations. The cell cycle-associated fluctuations in STPK13 mRNA abundance could be due to cell cycle-associated regulation of transcription or to regulation of posttranscriptional processes of either mRNA production or decay. To investigate this issue, nuclei were isolated from serum-starved (0-h, G_0) cells and from cells 6 h

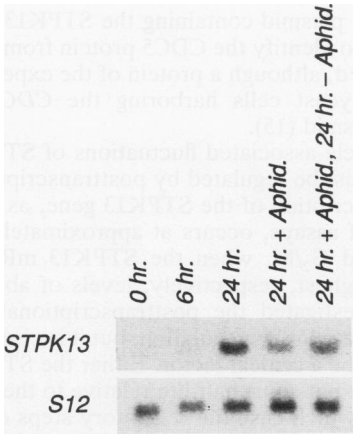

FIG. 7. Northern blot analysis of STK13 mRNA in NIH 3T3 cells arrested in S phase. Five flasks of NIH 3T3 cells were serum starved for 48 h and restimulated with serum as described in the legend to Fig. 5. Six hours after stimulation, aphidicolin was added to two flasks. Twenty-four hours later, the aphidicolin-containing medium in one of the flasks was replaced with aphidicolin-free medium. Cells were harvested for RNA analysis at the time of serum stimulation (0 hr.), at the time of aphidicolin addition (6 hr.), 24 h following aphidicolin addition (24 hr. and 24 hr. + Aphid.), and 24 h after removal of aphidicolin from the growth medium (24 hr. + Aphid, 24 hr. – Aphid.). Parallel flasks of cells were used to monitor [*methyl*-^{3}H]thymidine incorporation into DNA at each of these times. Levels of incorporation at 0, 6, and 24 h were, respectively, 55, 680, and 4,740 cpm in cultures that did not receive aphidicolin. After 24 h of aphidicolin treatment, 20 cpm was incorporated; 2,200 cpm was incorporated 24 h after aphidicolin was removed. Total cell RNA was extracted from each cell sample, and 20 μg of each RNA was subjected to Northern blot analysis as described in Materials and Methods. The Northern membrane was first hybridized with radiolabeled mouse STPK13 cDNA sequences and subsequently rehybridized with radiolabeled cDNA sequence encoding the mouse S12 ribosomal protein.

(G_1) and 24 h (G_2 and M) after serum stimulation and allowed to synthesize RNA in the presence of [α-^{32}P]UTP. Equal numbers of counts per minute of RNA from each sample, representing the RNA synthesized by 3×10^7 0-h nuclei, 2×10^7 6-h nuclei, and 1.5×10^7 24-h nuclei, were hybridized to slot blots of STPK13 cDNA. Figure 8 shows that the 0-h (G_0) nuclei synthesized approximately twice as much STPK13 RNA as either the 6-h (G_1) or 24-h (G_2 and M) nuclei, which synthesized approximately equal amounts as one another. If the hybridization signals are corrected for amount of STPK13 RNA synthesized per nucleus, the signals from all three samples of nuclei are approximately equal. The hybridization signal from S12 RNA, which was used as a control, was approximately equal in all three samples. The critical observations are that both the 0-h (G_0) and the 6-h (G_1) nuclei synthesized STPK13 RNA, and the 24-h (G_2 and M) nuclei did not show an increased synthesis compared with the 0-h (G_0) and 6-h (G_1) nuclei. In contrast, the experiments of Fig. 5 to 7 all show that STPK13 mRNA does not accumulate in G_0 or in G_1 but does accumulate to a maximum in G_2/M. The absence of STPK13 mRNA during G_0 and G_1, therefore, is not due to the absence of STPK13 gene transcription but rather must be due to posttranscriptional events which regulate its rate of formation or its rate of decay or both.

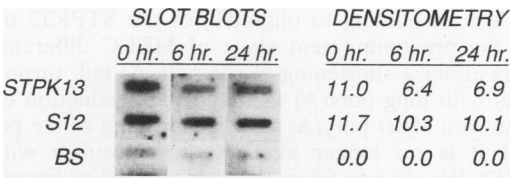

FIG. 8. Synthesis of STPK13 RNA in nuclei isolated from serum-starved and from serum-restimulated NIH 3T3 cells. NIH 3T3 cells were serum starved for 48 h and restimulated with serum as described in the legend to Fig. 5. At the time of serum addition (0 hr.) and at 6 h (6 hr.) and 24 h (24 hr.) later, nuclei were isolated and used for run-on RNA synthesis in the presence of [α-^{32}P]UTP as described by Greenberg and Ziff (6); 4×10^7 cpm of each resulting radiolabeled RNA sample, representing the RNA synthesized by 3×10^7, 2×10^7, and 1.5×10^7 nuclei, respectively, from the 0-, 6-, and 24-h samples, were hybridized to slot blots loaded with 20 μg of Bluescript plasmid DNA without any insert (BS), with the STPK13 cDNA insert (STPK13), or with the S12 ribosomal protein cDNA insert (S12). Conditions of hybridization and subsequent treatment of the blots are given in Materials and Methods. The blots were exposed to X-ray film, and the radioautograph was scanned with a laser densitometer. Values at the right are units of absorbance at the peak heights. Background absorbance of the Bluescript plasmid without any insert was set to 0.0.

DISCUSSION

We identified a poly(A)-terminated mRNA for a putative protein kinase whose abundance changes substantially during the precommitment stage of MELC terminal erythrodifferentiation. Further studies demonstrated that this mRNA, which we termed STPK13, is not restricted to erythroid cells but is present in a variety of actively growing cells, is absent from nongrowing cells, and is regulated in abundance during the cell cycle. The putative protein kinase encoded by the STPK13 mRNA appears to be the homolog of the *Drosophila* protein kinase encoded by the *polo* gene and that encoded by the *S. cerevisiae CDC5* gene, both of which are required for proper cell division (10, 15, 17, 24).

Regulation of the STPK13 mRNA in precommitment MELC is different from that during normal cell cycling. In MELC, the amount of the STPK13 mRNA that binds to oligo(dT)-cellulose decreases approximately sixfold during the precommitment period, but the body of the mRNA remains constant in abundance during the entire induction period. We interpret the oligo(dT) binding characteristics of the STPK13 mRNA to be an indirect measure of the status of its poly(A) tail. During the precommitment stage of MELC induction, that status changes, resulting in a loss of binding. No loss of oligo(dT) binding was observed for the STPK13 mRNA in mutant MELC that fail to commit to terminal differentiation in response to conventional inducers, nor in wild-type MELC for 11 other poly(A)-terminated mRNAs that we assayed, including that encoding the small ribosomal protein S12, which we used as a control in the studies reported here. The decline in abundance of STPK13 poly(A)-terminated mRNA must, therefore, be a specific event associated with MELC commitment to terminal differentiation and must involve some change in the poly(A) tail but not in the body of the mRNA. In contrast, the abundance of the body of the STPK13 mRNA periodically changes in actively growing cells, in synchrony with the cell cycle. It accumulates to a maximum during the G_2 and M phases, declines to a nearly undetectable level following mitosis and throughout G_1 phase, and then begins to accumulate again during S phase.

The loss of binding to oligo(dT) of the STPK13 mRNA during the precommitment stage of MELC differentiation suggests either a shortening of the poly(A) tail, turnover of mRNAs with long poly(A) tails and the production of new mRNAs with short poly(A) tails, or masking of the poly(A) so that it is no longer available to hybridize with the oligo(dT). We do not favor the last possibility because all RNA samples were extensively extracted with phenol and chloroform, which should have removed any noncovalently associated protein that might sequester the poly(A), and immediately before application to oligo(dT) columns, RNA samples were heat denatured to disrupt bimolecular hydrogen bonds with other RNA molecules that might already occupy the poly(A) and prevent it from binding to the oligo(dT). Current data do not allow a distinction between the first two possibilities, but we favor poly(A) shortening because it has been observed for several other mRNAs. Examples of different mRNAs that exist as forms that do and do not bind oligo(dT)-cellulose have been reported (reviewed in references 2 and 13). Usually these two forms exist differentially at different stages of development of the organisms in which they were identified. Many but not all mRNAs that do not bind to oligo(dT), and presumably have either a short or no poly(A) tail, do not translate well. A particularly compelling example was demonstrated during early mouse development; in this case, regulation of poly(A) length by a deadenylating activity coordinately inhibits translatability of the mRNA for tissue-type plasminogen activator (11). We have not yet investigated whether the change that we observe in the STPK13 poly(A) tail influences its translation, nor have we investigated the mechanism by which it occurs.

The periodic accumulation and loss of the STPK13 mRNA during the cell cycle can be reconciled with the cell cycle timing of its putative function. Mutations at the *Drosophila polo* locus cause abnormal mitotic divisions (17, 24), and Fenton and Glover (5) have recently demonstrated that the *polo* protein kinase activity peaks during late anaphase/telophase in *Drosophila* syncytial embryos. Therefore, the *polo* protein kinase must function in the process of mitosis. Likewise, the yeast *CDC5* mutation blocks nuclear division (10). The observed accumulation of the STPK13 mRNA to a maximum during the G_2/M phase of the cell cycle would make available the maximum number of translational templates, with the presumed consequence that a maximum number of STPK13 protein kinase molecules would be available just preceding or during mitosis, the time when it is expected to function. The observed absence of the STPK13 mRNA following mitoses and throughout the G_1 phase would have the consequence that no new STPK13 protein kinase molecules would be synthesized at these times of the cell cycle. It has recently been demonstrated that the abundance of the *CDC5* mRNA fluctuates throughout the *S. cerevisiae* cell cycle similarly to the fluctuation reported here for the STPK13 mRNA (15), a behavior which, in addition to protein sequence identity, supports the idea that the STPK13 protein is the mouse homolog of the *S. cerevisiae CDC5* protein kinase. If the abundances of the STPK13 and the CDC5 proteins also fluctuate during the cell cycle in concert with the fluctuations in abundances of their mRNAs, then at least a part of the regulation of these protein kinases must occur by events that control their mRNA abundances. We are currently precluded from examining the levels of the STPK13 protein because we have been unable to detect it by Western blot (immunoblot) analysis, although we have detected a protein of the expected size in cells transfected with an expression plasmid containing the STPK13 cDNA. Similar attempts to identify the CDC5 protein from *S. cerevisiae* have also failed, although a protein of the expected size was identified in yeast cells harboring the *CDC5* gene in a multicopy plasmid (15).

The cell cycle-associated fluctuations of STPK13 mRNA abundance must be regulated by posttranscriptional events, because transcription of the STPK13 gene, as measured by nuclear runoff assays, occurs at approximately equal rates during G_1 and G_2/M, when the STPK13 mRNA is at its lowest and highest, respectively, levels of abundance. We have not investigated the posttranscriptional events that might participate in this regulation, but it is clear that mRNA half-life must be a critical factor. Either the STPK13 mRNA has a constant but short half-life relative to the length of the cell cycle, in which case the regulatory steps controlling its abundance must take place during some posttranscriptional process of mRNA formation or during delivery to the cytoplasm, or else the STPK13 mRNA half-life varies during the cell cycle, in which case it must be targeted for destruction at the end of mitosis and during the G_0 and G_1 phases. Current data do not allow a distinction between these possibilities, and there is no reason at present to exclude a combination of them.

Whether the loss or shortening of the STPK13 mRNA poly(A) tail that we observed indirectly during the MELC precommitment period also participates in the regulation of the abundance or function of this mRNA during normal cell cycling remains an intriguing but uninvestigated possibility. All terminal differentiation is accompanied by a loss of cell division. If, as has been demonstrated for several mRNAs (reviewed in references 2 and 13), the loss of the STPK13 mRNA poly(A) tail during the MELC precommitment period reduces its translational efficiency, the result would be a reduction in the cellular abundance of its encoded protein kinase which, by analogy with the *Drosophila polo* protein kinase and the *S. cerevisiae CDC5* protein kinase, would be required for mitosis. This in turn, would contribute to limiting cell divisions as commitment to terminal differentiation ensues.

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