Alternative Splicing of STY, a Nuclear Dual Specificity Kinase*

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The LAMMER subfamily of kinases has been conserved throughout evolution, and its members are thought to play important roles in the regulation of cellular growth and differentiation programs. STY is a murine LAMMER kinase which has been implicated in the control of PC12 cell differentiation. Multiple transcripts are derived from the Sty gene, and their relative abundance is developmentally regulated. Alternative splicing of the primary Sty transcript generates mRNAs encoding full-length catalytically active (STY) and truncated, kinase-deficient polypeptides. Both STY and its truncated isoform, STYT, are localized in the nucleus and are capable of heterodimerizing. We also demonstrate that STY functions as a dual specificity kinase in mammalian cells.

The Sty gene encodes a member of the recently discovered family of dual specificity kinases (1, 2). To date, at least 18 distinct genes encoding dual specificity kinases have been identified in the genomes of yeast and mammals (for reviews, see Douville et al. (3) and Lindberg et al. (4)). These kinases, expressed either as bacterial products or isolated from mammalian cells, have the ability to autophosphorylate on serine, threonine, and tyrosine residues. Two of the best studied of these enzymes are the yeast wee1 gene, a regulator of progression through the cell cycle (5), and MEK (MAPK/ERK kinase), believed to be a key molecule in mitogen-stimulated signaling pathways (6). We have previously reported the cloning and sequencing of three novel cDNAs encoding STY mRNAs (1). The STY kinase contains an amino acid motif, LAMMER, found in kinase subdomain X (7), a feature which is shared with at least eight other dual specificity kinases expressed in humans, mice, plants, and insects (7–9). The LAMMER motif containing protein kinases appears to be conserved throughout evolution suggesting that these enzymes may play important roles in the control of cellular growth and differentiation. Indeed, the Doa kinase, a Drosophila gene product, is critical to the development of the fly embryo and affects eye differentiation in the adult (7, 10). The AFC1 kinase gene of Arabidopsis thaliana can complement yeast signal transduction mutants via activation of the transcription factor STE12 (8). In mammalian cells, the physiological function of the LAMMER kinases is largely unknown although overexpression of the STY kinase in PC12 cells appears to initiate their differentiation into neural derivatives possibly through activation of a protein kinase cascade (11). As has been observed with other members of the LAMMER kinase family, the STY gene appears to express several differentially processed transcripts. To gain a full understanding of the physiological role of the STY kinase, we felt it important to identify and biochemically characterize all its gene products. We report here the cloning and sequencing of these novel STY cDNAs. Two of these cDNAs are derived from incompletely processed transcripts which accumulate in the nucleus in a developmentally regulated fashion. The third cDNA encodes a truncated polypeptide (STYT), which like the full-length STY kinase is found in the nucleus and contains domains which facilitate dimerization. Furthermore, we present evidence that the STY kinase is able to phosphorylate serine, threonine, and tyrosine residues when expressed in mammalian cells.

MATERIALS AND METHODS

Construction of cDNA Library—An L1210 leukemia cell line cDNA library was constructed in gt10 from size-selected poly(A)" RNA. Poly(A)" RNA was fractionated through a 2–10% sucrose gradient in 85% formaldehie, 1 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.2% SDS. Fractions were run at 200,000 × g for 20 h at 20°C. Following centrifugation, 0.4-ml fractions were collected, and aliquots were analyzed by Northern blotting using a Stx cDNA probe. Fractions containing RNA larger than 1.8 kb were pooled and precipitated with ethanol. cDNA was generated with 5 μg of size-selected poly(A)" RNA using the SuperScript cDNA synthesis system (Life Technologies, Inc.) and was subsequently size-selected for products larger than 1 kb by gel filtration (Sephараse 4B, Pharmacia Biotech Inc.).

Isolation of cDNA Clones—The gt10 library was screened using a full-length Stx cDNA probe. From a screen of 200,000 plaques, 18 positives were identified. Six were chosen at random for further analysis. To determine the size of the inserts, PCR was employed to amplify cDNA using gt10 and Stx specific primers. Briefly, pairs of gt10/Stx primers were used to amplify DNA from the primary plaques of the library. Primer pairs were gt10 forward ("agcaagttcagcctggttaag") and Stx619F ("tccacacatttttccagga") or gt10 reverse ("dttatgattttcttccagga") and Stx619F. 50-μl PCR reactions were performed with 50 pmol of each primer, 0.2 mM concentration of each dNTP, 1.25 units of Taq DNA polymerase in 50 mM KCl, 10 mM Tris-HCl, pH 8.4, 2.5 mM MgCl2, and 0.2 mg/ml gelatin. PCR conditions were 94°C, 40 s; 50°C, 60 s; 72°C, 2 min for 30 cycles. Amplified products were resolved by agarose gel electrophoresis and ethidium bromide staining. Two

1 The abbreviations used are: kb, kilobase(s); bp, base pair(s); mAb, monoclonal antibody; Tyr(P), phosphotyrosine; E, exon; I, intron; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcription polymerase chain reaction; MOPS, 4-morpholinepropanesulfonic acid.
clones yielding inserts of approximately 2.6 kb were plaque-purified by standard procedures (12).

Sequence of cDNA Clones—The cDNA inserts were subcloned into the KpnI site of the plasmid pGEM-4 (Promega). Double-stranded DNA sequencing was carried out using the dideoxy chain termination method (13). Full-length sequence of the cDNA insert was achieved using oligo(dT)-primed reverse transcription with cDNA synthesized from total RNA from the liver of a 3-week-old Sprague-Dawley rat. Northern Hybridization Analysis—Total RNA was prepared from cells or tissues as described by Chirgwin et al. (14). Poly(A) + RNA was selected by passage of total RNA through oligo(dT)-cellulose columns as described by Jacobson (15). To isolate nuclear and cytoplasmic RNA, NIH 3T3 cells (3 × 106) were resuspended in 1 ml of lysis buffer (10 mM Tris-HCl, pH 8.0, 140 mM KCl, 5 mM MgCl2, 0.5 mM dithiothreitol, 0.5% Nonidet P-40) and lysed in a Dounce homogenizer. Nuclei were removed from the cytoplasmic fraction by centrifugation for 5 min at 1200 × g. Total nuclear and total cytoplasmic RNA was isolated from these fractions as outlined above. Aliquots of total RNA (20 μg) were electrophoresed through 1% agarose gels containing 1% formaldehyde, 40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA, transferred to Hybond N membrane (Amersham), and UV-cross-linked as described by Southern (16). The hybridization was carried out in 50% formamide, 0.25 M NaCl, 0.05 M sodium citrate, 5 × Denhardt’s solution (1%/w/v) Ficol, 1%/w/v polyvinylpyrrolidone, 1%/w/v bovine serum albumin, 0.5% SDS, 150 μg/ml sheared herring sperm DNA, with a random-primed [32P]-labeled cDNA probe at 42°C. Blots were washed in 0.2 x SSC, 1% SDS at 65°C. The positions of 18S and 28S RNA were determined by ethidium bromide staining of the agarose gel.

RT-PCR Analysis and Cloning of Sty—Total RNA from P19 cells or mouse tissues was used to synthesize oligo(dT)-primed first strand cDNA with SuperScript RNaseH− reverse transcriptase (Life Technologies, Inc.) as per the manufacturer’s instructions. 1–2 μl of a 20-μl cDNA synthesis reaction was subsequently used in a PCR amplification. 50-μl reactions were performed containing 20 pmol of primer Sty619F, 20 pmol of primer Sty93R (5′cggatccatgagacattcaaagagaact3′) and 1.5 units of Taq DNA polymerase for 30–40 cycles under the conditions described above. Sty-cDNA was generated by RT-PCR using primers Sty93R(5′cggatccatgagacattcaaagagaact3′) and primer Sty1539F(5′TTTCCGGGTGTTTGTGATTGGGT3′). Amplified products were resolved by electrophoresis through a 1.5% agarose gel and ethidium bromide staining. Sequencing of RT-PCR Products—Amplified PCR products were gel-purified and cloned directly into the pCRII plasmid (Invitrogen) (7R/9E10 hybridoma cultures, supernatant at 1:50–1:100). Immunoblot’s were resolved by boiling. Samples were resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membrane, and exposed to Kodak XAR-5 x-ray film.

For immunoblots, membranes were blocked in 5% Blotto in TBST (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.05% Tween 20). Anti-Myc immunoblotting was performed using a mouse monoclonal mouse anti-Myc (9E10) and a rabbit polyclonal antibody followed by enhanced chemiluminescence (Amersham) and exposure to Kodak XAR-5 x-ray film. Phosphoamino acid analysis was performed as described previously (19).

Immunofluorescence of M-STY in COS-1 Cells—Following transfection of pCE constructs into COS-1 cells, approximately 7.5 × 104 cells were plated onto gelatin-coated coverslips. 22–24 h post-transfection, cells were washed with phosphate-buffered saline and fixed in −20°C methanol for 10 min at −20°C. Following rehydration of cells in phosphate-buffered saline, anti-Myc mAb (9E10) was incubated for 1 h at 4°C. Following washing the immunofluorescence buffer, and proteins were eluted with sample buffer. Bound proteins were analyzed by SDS-PAGE, transferred to nitrocellulose, and subsequently immunoblotted with anti-Myc mAb.

RESULTS

Sequence Analysis of the Developmentally Induced Sty Transcripts Reveals Introns—Northern blot analysis revealed that the L1210 cell line expressed three distinct species of Sty mRNA corresponding in size to the developmentally regulated mRNA species identified previously in P19 cells and other cell lines (data not shown, Ref. 1, and Fig. 2). In order to determine if these transcripts encode different isoforms of the Sty kinase, we cloned the larger of these mRNA species. Using size-selected mRNA isolated from L1210 cells, we prepared cDNAs

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Fragment with XhoI. All mutations were confirmed by sequencing.

The Myc epitope in these constructs is immunoreactive with the monoclonal antibody (mAb) 9E10 in immunoprecipitation/immunoblot analysis (18).

Sty was also subcloned into the bacterial expression vector pGEX-3X (Pharmacia) to generate the fusion protein GST-STY. The 1.7-kb Sty fragment was isolated from pCRII/Sty by PCR amplification as described above. This blunt/EcoRI fragment was subcloned into pGEX-3X (BamHI, bluntend, followed by EcoRI digestion). Sty was confirmed to be in-frame with GST by sequencing.

COS-1 Cell Culture and Transfection—COS-1 cells were maintained in a minimal essential medium supplemented with 10% calf serum. For transfection, COS-1 cells were trypsinized, counted, and resuspended in serum-free–minimal essential medium at 2–3 × 106 cells in 0.5 ml. 20 μg of the appropriate plasmid DNA was added, and the cells were chilled on ice for 10 min. Cells were electroporated using a Gene Pulser (Bio-Rad) at 220 V and 960 μF following which the cells were left at room temperature for 15 min. Transfectants were then plated in α-encoded essential medium supplemented with 10% calf serum in the absence or presence of 50 μg sodium vanadate. Cells were harvested 24 h later.

Immunoprecipitation/Immunoblot Analysis—Transfected COS-1 cells were lysed in immunoprecipitation buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 2 mM NaF, 2 mM sodium pyrophosphate, 500 μM sodium vanadate, 200 μM phenylmethylsulfonyl fluoride, 20 μM chelating agents). Ammonium sulfate precipitation of the p910 hybridoma supernatant at 1:50–1:100. Immunoblots were visualized with horseradish peroxidase-conjugated goat anti-mouse antibody followed by enhanced chemiluminescence (Amersham) and exposure to Kodak XAR-5 x-ray film. Phosphoamino acid analysis was performed as described previously (19).

Immunofluorescence of M-STY in COS-1 Cells—Following transfection of pCE constructs into COS-1 cells, approximately 7.5 × 104 cells were plated onto gelatin-coated coverslips. 22–24 h post-transfection, cells were washed with phosphate-buffered saline and fixed in −20°C methanol for 10 min at −20°C. Following rehydration of cells in phosphate-buffered saline, anti-Myc mAb (150 in phosphate-buffered saline containing 0.3% Triton X-100) was added followed by fluorescein iso-thiocyanate-conjugated anti-mouse secondary antibody (1:20, DAKO).

GST-STY Binding Columns—GST-STY binding reactions were carried out essentially as described (20). Briefly, bacterially expressed GST or GST-STY was coupled to glutathione-Sepharose 4B beads (Pharmacia) in immunoprecipitation buffer. Approximately 10 μg of GST or GST-STY coupled to beads was used in each binding experiment. COS-1 cell lysates containing M-STY, M-STY + M-Tik, or M-Tik were mixed with GST or GST-STY beads. Incubations were carried out for 2 h at 4°C on a rotating platform. The beads were then washed with immunoprecipitation buffer, and proteins were eluted with sample buffer. Bound proteins were analyzed by SDS-PAGE, transferred to nitrocellulose, and subsequently immunoblotted with anti-Myc mAb.

By blutide Sequence Accession Numbers—The GenBank accession numbers are U11094 and U12209 for the Sty6 and Sty7 cDNAs, respectively.
from mRNA species larger than 1.8kb (see "Materials and Methods"). Our initial screen of 200,000 recombinants resulted in 18 positive clones, one of which (2.6 kb in size) was sequenced fully. This 2.6-kb cDNA clone contained regions of sequence identity with the *Sty* 1.8-kb embryonic transcript (hereafter referred to as *Sty* 1.8), including the 5' end, interspersed with regions of non-identity. The intervening sequences did not, however, maintain the open reading frame as they contained stop codons in all reading frames (see Fig. 1).

Further analysis revealed 5' and 3' splice sites at the ends of FIG. 1. Nucleotide and predicted amino acid sequences of the *Sty* 5.6 partial cDNA and *StyT* cDNA. The amino acid sequence is derived from that predicted from the *Sty* 1.8 cDNA (1). A, *Sty* 5.6 partial cDNA. Predicted intron/exon boundaries are indicated in boldface. The alternatively spliced exon is underlined, and consensus amino acids found in kinase subdomains are highlighted and indicated by Roman numerals. Amino acids indicated by an asterisk are encoded by codons split between two adjacent exons. Exons (E) and introns (I) are labeled as follows: EA, nucleotides 1–456; IA, nucleotides 457–1326; EB, nucleotides 1327–1417; IB, nucleotides 1418–1811; EC, nucleotides 1812–1995; IC, nucleotides 1996–2082; ED, nucleotides 2083–2249; ID, nucleotides 2250–2572.

B, *Sty* 1.7 (StyT) cDNA.

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Fig. 1. Nucleotide and predicted amino acid sequences of the Sty5.6 partial cDNA and Sty T cDNA. The amino acid sequence is derived from that predicted from the StyL.8 cDNA (1). A, Sty5.6 partial cDNA. Predicted intron/exon boundaries are indicated in boldface. The alternatively spliced exon is underlined, and consensus amino acids found in kinase subdomains are highlighted and indicated by Roman numerals. Amino acids indicated by an asterisk are encoded by codons split between two adjacent exons. Exons (E) and introns (I) are labeled as follows: EA, nucleotides 1–456; IA, nucleotides 457–1326; EB, nucleotides 1327–1417; IB, nucleotides 1418–1811; EC, nucleotides 1812–1995; IC, nucleotides 1996–2082; ED, nucleotides 2083–2249; ID, nucleotides 2250–2572. B, StyL.7 (Sty T) cDNA.
the intervening regions suggesting the presence of intronic sequences within an incompletely spliced transcript. The 2.6-kb cDNA did not contain the entire predicted Sty open reading frame, but rather contained four exons (including an exon with the predicted initiating methionine), three complete introns (with 5' and 3' splice sites), and one partial intron (with a 5' splice site). To determine from which mRNA species the cDNA had been cloned, Northern blots were probed with the cDNA regions encompassing individual introns (data not shown). The results of these experiments are depicted schematically in Fig. 2. Probes derived from intron A (IA), or intron B (IB), hybridized to the 5.6-kb and 3.2-kb transcripts (hereafter called Sty5.6 and Sty3.2, respectively), while probes from intron C (IC) and D (ID) hybridized to Sty5.6 but not Sty3.2. These data indicated that the 2.6-kb cDNA clone represented a partial 5' clone of the Sty5.6 RNA species. Since probes corresponding to introns A and B hybridized to Sty3.2, it is likely that this mRNA species is also an incompletely spliced transcript. Thus, both the larger 5.6- and 3.2-kb transcripts contain intron sequences and arise as a result of incomplete and/or alternative splicing. The intron/exon boundaries of the 2.6-kb cDNA clone are indicated in Table 1.

Sty Is Alternatively Spliced—Alignment of the amino acid sequence of STY with that of the reported human homologue (hCLK (21)) using the Lipman-Pearson algorithm (22) revealed very high homology (83% identity over 482 amino acid overlap). However, there is a stretch of 30 amino acids contained in STY that is absent within hCLK (Fig. 3A). Sequence analysis indicated that these 30 amino acids are encoded within the second exon (exon B) of the 2.6-kb cDNA clone (see Figs. 1 and 3B). This suggested that exon B may be alternatively spliced. The Sty cDNA originally isolated (1) would encode the isoform containing exon B, whereas the hCLK cDNA (21) would encode the isoform lacking this exon. Consistent with this idea, the Sty1.8 transcript often appears as a doublet upon Northern blotting suggesting the presence of a fourth transcript (data not shown, Ref. 1, and Fig. 2).

To investigate this possibility, we performed reverse transcriptase coupled with PCR amplification (RT-PCR) to amplify the entire coding sequence of Sty (nucleotide position 7–1539, data not shown) from P19 cell-derived cDNA. Two DNA species were amplified, the sizes of which were consistent with alternative splicing of exon B (data not shown). To verify the identity of the PCR products, the DNA was isolated, reamplified, and subcloned into the pCR11 vector. Sequencing revealed that the larger amplification product corresponded to full-length Sty, whereas the smaller lacked exon B (Fig. 1). This result was observed with cDNA derived from P19 cells (see P19 lane, Fig. 4) suggesting that the alternatively spliced transcript lacking exon B (Sty1.7) arises from a mRNA that co-migrates with the 1.8-kb transcript.

Expression of Sty1.7 and Sty1.8 was assayed by RT-PCR amplification of the region encompassing exon B from cDNA of several mouse tissues (Fig. 4). All tissues tested contained both Sty1.7 and Sty1.8 transcripts. A PCR product of approximately 1 kb in size was also reproducibly amplified from all tissues, the size of which is consistent with that of a splicing interme-
The coding sequence is capitalized, while the noncoding sequence is in small letters. The numbering of nucleotide positions is relative to that of Sty1.8 (1). Intron/exon boundaries indicated by an asterisk were determined by sequencing genomic DNA from the Sty locus. ND, not determined.

### Table 1

| S' SPlice Site | INTRON SIZE | 3' SPlice SITE |
|----------------|-------------|----------------|
| Intron A       | "GGG:gtatga"| -870bp         |
| Intron B       | "GAT:gtatag"| -394bp         |
| Intron C       | "TTT:gtatag"| -86bp          |
| Intron D       | "ACT:gtatag"| >320bp         |
| Intron 1*      | ND          | ND             |
| Intron 2*      | "CAG:gtatag"| -600bp         |
| Consensus      | "AG:gtatag" | >60bp          |

A

**Mouse**

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HHTSQHSHGKSHRRKSRSVDEDEEGLICQSGDVLSARYEIVDTIL
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**Human**

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HSTSHRRSHG------------------------DEIVDTIL
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B

Fig. 3. Schematic representation of the alternative splicing of exon B. A, comparison of the amino acid sequence surrounding exon B of STY (mouse) with that of the human homologue (hCLK). The sequence encoded by exon B in mouse which is absent in the human cDNA is denoted by the dashed line. Amino acid positions indicated are relative to those predicted for STY (1) and hCLK (17). Identical residues are identified by bars, and conservative substitutions by dots. B, the nucleotide sequence of the intron/exon boundaries surrounding the alternatively spliced exon is shown between the predicted splicing products. Splicing in of exon B would result in production of full-length STY. Splicing out of exon B results in a frameshift which introduces a premature stop codon (tag, indicated by a period). The resulting protein (STY) is truncated prior to the catalytic domain.

However, the protein product of hCLK, which lacks exon B, is predicted to be catalytically active. This apparent contradiction suggested that either the sequence encompassing exon B is absent in the human genome and that in its absence the gene has evolved to maintain a catalytically active molecule or, alternatively, the hCLK cDNA is the homologue of Sty1.7 and

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The Sty1.7 mRNA, generated by alternative splicing of exon B, should encode a protein homologous to the hCLK protein product. The protein product from the murine Sty1.7 transcript is predicted to be truncated and catalytically inactive (we have termed this product STYT for truncated STY) (see Fig. 3B).
should encode a truncated molecule. To investigate this further, a human liver cDNA library (gift of Dr. D. A. Gray) was screened with a full-length Sty cDNA probe. From an initial screening of 200,000 recombinants, 30 clones were identified. Of these, 10 were picked at random and analyzed by PCR to determine if they contained the exon A-exon C region. One positive clone was analyzed further, and the sequence of this boundary indicated that it was the human homologue of StyL.7 and would encode a truncated protein product (data not shown). The human cDNA, as published by Johnson and Smith (21), contains an additional G residue at this boundary which would maintain the open reading frame through the catalytic domain. The absence of this residue in our mouse and human cDNAs causes a frameshift to occur which introduces a stop codon (Fig. 3B).

Expression of STY Protein in Mammalian Cells—The Sty cDNA encodes a 57-kDa polypeptide, whereas the StyT cDNA encodes a 16.3-kDa truncated polypeptide lacking the catalytic domain. In order to detect these protein products, we tagged the STY and STYT polypeptides with a six-repeat human Myc epitope and expressed these fusion proteins in COS-1 cells. The epitope tag added 89 amino acids to the amino-terminal end, increasing the molecular mass by approximately 10 kDa. COS-1 cells transfected with the pECE vector alone showed no proteins detectable by anti-Myc immunoblotting (Fig. 5A, lane 1). Transfection of pECE vectors encoding Myc-tagged STY (M-STY) or STYT (M-STYT) cDNAs demonstrated anti-Myc immunoreactive proteins of approximately 75 kDa and 34 kDa, respectively (Fig. 5A, lanes 2 and 4) corresponding to full-length and truncated STY kinases.

STY Protein Exhibits Dual Specificity Kinase Activity in Vitro and in Vivo—STY expressed in bacteria has dual specificity kinase activity (1). To determine if STY expressed in mammalian cells has similar dual specificity kinase activity, we used an immune complex kinase assay. Cells transfected with the M-STY plasmid expressed a 75-kDa phosphoprotein as detected in an anti-Myc mAb immune complex kinase assay (Fig. 5B, lane 2). Phosphoamino acid analysis of the M-STY phosphoprotein revealed substantial phosphoserine, phosphothreonine, and phosphotyrosine (Tyr(P)) (Fig. 5C). It remained possible that a co-precipitating kinase was responsible for phosphorylation of some or all of the sites on M-STY. To address this problem, a catalytically inactive mutant of M-STY, M-STY\(^{K190R}\) was generated. Oligonucleotide-directed PCR mutagenesis was used to change the invariant lysine residue 190 in subdomain II of the catalytic domain to an arginine residue (K190R). While expression of this mutant could be readily detected by immunoblotting COS-1 cell extracts (Fig. 5A, lane 3), M-STY\(^{K190R}\) showed no kinase activity or evidence of phosphorylation (Fig. 5B, lane 3). This result demonstrates that STY protein expressed in mammalian cells exhibits dual specificity kinase activity. To test its in vivo phosphorylation state, M-STY was transfected into COS-1 cells, immunoprecipitated with either anti-Myc mAb or anti-Tyr(P) mAb, and then subsequently immunoblotted with anti-Myc mAb (Fig. 6). Cells grown in the absence or presence of vanadate, a phosphotyrosine phosphatase inhibitor, showed similar levels of anti-Myc immunoprecipitable M-STY (Fig. 6, lanes 1 and 2). In the absence of vanadate, only a fraction of the M-STY could be immunoprecipitated with anti-Tyr(P) mAb (Fig. 6, lane 3). In the presence of vanadate, however, similar amounts of M-STY could be immunoprecipitated with anti-Tyr(P) mAb (Fig. 6, compare lanes 2 and 4). As expected, the M-STY\(^{K190R}\) mutant did not react with the antibody directed against phosphotyrosine (data not shown). These results were corroborated by incubating transfected COS-1 cells with \(^{32}P\)orthophosphate and performing phosphoamino acid analysis on labeled M-STY protein. As with the in vitro kinase assay, all three hydroxylamine acids were phosphorylated when M-STY was labeled in vivo (data not shown).

Nuclear Localization of STY Protein—The subcellular localization of M-STY in transfected COS-1 cells was determined by indirect immunofluorescence with the anti-Myc mAb. M-STY
expressed in COS-1 cells was nuclear with undetectable cytoplasmic staining (Fig. 7b). Deletion of the first 60 amino acids of STY (M-STY\(^{\Delta 60\text{II}}\)), which contain the predicted nuclear localization signal, directed widespread cytoplasmic expression of this polypeptide (Fig. 7c). Nuclear staining was not eliminated, suggesting that there may be multiple nuclear localization signals in STY. M-STY\(^{\Delta 60\text{II}}\) expressed in COS-1 cells as assessed by immunoblotting, retained in vitro catalytic activity (Fig. 5A, lane 5, Fig. 5B, lane 5, respectively). The mutants, M-STY\(^T\) and M-STY\(^{K190R}\), were also targeted to the nucleus (Fig. 7d and data not shown) demonstrating that catalytic activity is not required for nuclear compartmentalization of STY.

**DISCUSSION**

Isoforms of several protein kinases have been predicted and identified based on cDNA cloning of multiple RNA transcripts (23-25). For instance, six transcripts, coding for at least two protein isoforms, are derived from the gene encoding the trkB neurogenic tyrosine kinase receptor (23). These trkB proteins, gp145\(^{\text{trkB}}\) and gp95\(^{\text{trkB}}\), have identical extracellular and transmembrane domains, but only gp145\(^{\text{trkB}}\) contains the cytoplasmic kinase domain. Similarly, the fibroblast growth factor tyrosine kinase receptor 1 gene generates several transcripts which encode receptor variants (24). One of these, a receptor-like molecule that lacks the transmembrane and kinase domains, is secreted and catalytically inactive.

The expression of the Sty gene is developmentally regulated at the level of transcript processing. Embryonic stem cells express two major transcripts (1.7 and 1.8 kb), whereas differentiated cells express two additional partially spliced mRNAs (3.2 and 5.6 kb). While the two larger mRNAs are sequestered in the nucleus and are unavailable for protein translation, the 1.7- and 1.8-kb mRNAs are capable of directing synthesis of a truncated and full-length STY protein, respectively. In general, unspliced primary transcripts are relatively short-lived with...
Amino acid comparison of STY with its human homologue, hCLK, revealed a 30-amino-acid insertion in STY. Isolation and sequencing of the St5.6 partial cDNA done revealed that this 30-amino acid segment was contained within a single exon. Alternative splicing of this exon would generate either the full-length product, STY (containing the exon), or a truncated polypeptide (STY†, lacking this exon) due to a frameshift which introduces a stop codon (see Fig. 3B). Johnson and Smith (21) suggested that the hCLK cDNA, which lacks the exon, coded for an active kinase. We have isolated a human Clk cDNA and sequenced it in this region. Our sequence indicated that the G

Clk2 (serine-arginine-rich) family of splicing factors. We are testing the possibility that hCLK2, hCLK3, and their truncated derivatives also associate with splicing factors.

One commonly accepted paradigm of kinase regulation is that dimerization is required for kinase activation and subsequent signal transduction (30, 31). Evidence for this idea includes the demonstration that catalytically inactive kinase mutants dominantly suppress wild type kinase activity (32). STY and STY† show identical patterns of expression and subcellular localization and can form heterodimers in vitro. We suggest that a dynamic interaction between STY and STY† may be involved in the regulation of the biological properties of STY and related kinases.

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