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STY, a Tyrosine-Phosphorylating Enzyme with Sequence Homology to Serine/Threonine Kinases

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We have cloned a novel kinase (STY) from an embryonal carcinoma cell line. Sequence analysis of the STY cDNA reveals that it shares sequence homology with serine/threonine-type kinases and yet the bacterial expression product of the STY cDNA appears to have serine-, threonine-, and tyrosine-phosphorylating activities. The predicted STY protein is highly basic and contains a putative nuclear localization signal. During differentiation, two new mRNAs were detected in addition to the embryonic transcript.

During embryogenesis there is stringent control over the processes of cellular differentiation and proliferation. Sophisticated networks of regulatory molecules have evolved to sense and transmit signals within and between differentiating cells. The recent demonstration that a number of developmental and cell cycle mutations map to genes encoding protein kinases (3, 4, 6, 11, 12, 27, 30, 32, 35) identifies these enzymes as important components of this regulatory machinery. In order to identify other kinases possibly involved in differentiation programs, we have chosen the P19 murine embryonal carcinoma (EC) cell line as a model system. When grown in the undifferentiated state, P19 cells appear to be morphologically, immunologically, and biochemically identical to the inner cell mass of the early mouse blastocyst (23). These cells are easily manipulated in vitro and can be induced to differentiate into a broad spectrum of cell types by using retinoic acid (RA) or dimethyl sulfoxide (DMSO) (16, 25).

Members of the protein tyrosine kinase (PTK) family have been identified in both vertebrate and invertebrate organisms. Although they have recently been identified in *Dictyostelium discoideum* (37) and yeast cells (13), apparently no PTKs are encoded by *Escherichia coli*. Bacteria therefore provide an ideal host for expression cloning of mammalian PTK cDNAs (21). By screening 500,000 recombinants with a monoclonal antibody against phosphotyrosine, we identified eight distinct positive clones including the ubiquitous PTK c-*fer* (28) and a number of novel kinases. One of these novel kinases, which we have designated STY, has homology with other kinases, primarily the serine/threonine type. Immunopurified bacterial expression products of the STY cDNA phosphorylated all three hydroxy amino acids in vitro. Judged by its pI of 8.9 and a putative nuclear localization signal in the amino-terminal portion of the molecule, STY is potentially a nuclear protein. Alternative forms of the STY transcript appeared upon differentiation of stem cells into their various derivatives.

Screening and isolation of cDNA clones. A P19 EC cell cDNA library constructed in λgt11 with P19 EC cell poly(A)$^+$ RNA was screened with the PY 20 monoclonal antiphosphotyrosine antibody (ICN Biochemicals) essentially as previously described (37). First- and second-strand cDNAs were prepared from the P19 EC cell poly(A)$^+$ RNA (7) with the cDNA Synthesis System Plus (Amersham) and were subsequently size selected for products larger than 1 kb by agarose gel electorphoresis.

Sequencing of cDNA clones. The cDNA inserts were cloned into the *Eco*RI site of the plasmid pTZ19R (Pharmacia). Nested deletions were generated with an Exo III-mung bean deletion kit (Stratagene). These deletion mutants were sequenced by the dideoxy chain termination method (34).

Bacterial expression of STY cDNA. The catalytic region of STY cDNA from the *Hin*dIII site (position 280 bp) to carboxyl-terminal *Eco*RI was subcloned into the *Sma*I site of the pATH2 expression vector (31, 36). *E. coli* JM103 harboring this construct was typically induced to express the *trpE*-STY fusion protein by growing the bacteria for 12 h in medium lacking tryptophan and containing indoleacrylic acid as described elsewhere (31). Alternatively, *E. coli* Y1090 was infected with the λgt11 STY clone and induced to express STY protein by the addition of 10 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to the bacterial medium for 4 h (20).

Phosphoamino acid and tryptic map analysis. Lysates were prepared from bacteria induced to express *trpE* or *trpE*-STY by sonication in 20 mM Tris hydrochloride (pH 8.0)–2 mM EDTA–100 mM NaCl–10 mM β-mercaptoethanol–0.075% Nonidet P-40. Lysates were cleared by centrifugation, and the supernatants were immunoprecipitated with either PY 20 or *trpE* (AB-1) monoclonal antibodies (from ICN Biochemicals and Oncogene Science Inc., respectively) in 10 mM Tris (pH 7.5)–150 mM NaCl–5 mM EDTA–1% Triton X-100–2 mM NaF–2 mM sodium pyrophosphate–500 μM ammonium vanadate–200 μg of phenylmethylsulfonyl fluoride per ml and assayed for kinase activity in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.1)–10 mM $MnCl_2$ for 30 min at 22°C. The reaction products were resolved by 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and the dried gels were exposed to Kodak XAR-5 film. Phosphoproteins were electroeluted for phosphoamino acid analysis by the method of Edwards et al. (9), and phosphotryptic maps were also prepared by the method of Edwards et al. (10).

Northern (RNA) blot analysis. Approximately 5 μg of poly(A)$^+$ RNA prepared by the method of Auffray and

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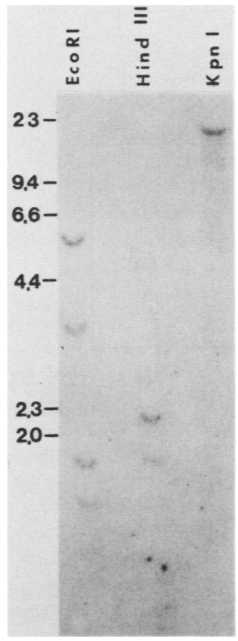

FIG. 1. Southern blot analysis of STY. BALB/c mouse DNA (20 μg) was restricted to completion with the enzymes indicated above the lanes and probed with the complete STY cDNA.

Rougeon (2) from tissues and cell lines were resolved on 1% agarose gels containing formaldehyde and then transferred and UV cross-linked to Hybond N membranes (Amersham) as described by the manufacturer. Hybridization was done in 50% formamide–5× Denhardt's solution (8)–6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5% dextran sulfate–0.1% SDS–150 μg of sheared salmon sperm DNA per ml with ^{32}P-labeled STY cDNA probe at 42°C prior to a high-stringency wash with 0.1× SSC–0.1% SDS at 65°C. The positions of 18S and 28S were determined by acridine orange staining of adjacent lanes of total RNA (see Fig. 5).

Southern blot analysis. BALB/c mouse DNA (20 μg) was isolated in the usual manner (33), restricted to completion with enzymes, fractionated on a 1% agarose gel, and transferred and UV cross-linked to Hybond N membrane (as described by the manufacturer) (Fig. 1). The autoradiogram shows the hybridization pattern of the full-length STY cDNA to the blot after high-stringency hybridization, as described in the legend to Fig. 4, omitting dextran sulfate from the hybridization solution.

Results and discussion. Of the five STY cDNAs identified, the longest clone was 1.8 kb (the size of the mRNA in P19 cells). This cDNA hybridized to a single *Kpn*I fragment of 20 kb on a Southern blot of mouse genomic DNA. Other restriction enzyme digests produced simple restriction patterns with total fragment sizes of about 12 kb, suggesting that the STY cDNA hybridizes to a single-copy gene (Fig. 1).

The amino-terminal region of the STY polypeptide predicted from the cDNA sequence (Fig. 2) has little homology to other known kinases and is probably not myristoylated since it lacks the common target, an amino-proximal glycine residue (17). Within the first 165 amino acids are a number of regions homologous to known nuclear proteins. In particular, there is a motif of basic amino acids (Lys-Arg-Lys-Lys-Arg) reminiscent of the nuclear localization signals of simian virus 40 large T antigen, p53, and the c-*abl* IV protein

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atcgtcgtaatcgtttgcagacttctcgccgtcgccttgtaagctttgtcttcg      54

                                    Met Arg His Ser Lys Arg      6
ccttgcaagctttgtcttcagggttggaaag atg aga cat tca aag aga    103

Thr Tyr Cys Pro Asp Trp Asp Glu Arg Asp Trp Asp Tyr Gly      20
act tac tgt cct gac tgg gat gaa aga gac tgg gat tat gga    145

Thr Trp Arg Ser Ser Ser Ser His Lys Arg Lys Lys Arg Ser      34
aca tgg aga agc agc agc agt cac aaa aga aag aag aga tca    187

His Ser Ser Ala Arg Glu Gln Lys Arg Cys Arg Tyr Asp His      48
cat agc agc gcc cgt gag caa aag cgc tgc agg tac gat cac    229

Ser Lys Thr Thr Asp Ser Tyr Tyr Leu Glu Ser Arg Ser Ile      62
tcc aaa acg aca gac agc tat tat ctg gaa agc aga tcc ata    271

Asn Glu Lys Ala Tyr His Ser Arg Arg Tyr Val Asp Glu Tyr      76
aat gag aaa gct tat cat agt cga cgc tat gtt gat gaa tac    313

Arg Asn Asp Tyr Met Gly Tyr Glu Pro Gly His Pro Tyr Gly      90
agg aat gac tac atg ggc tac gag cca ggg cat ccc tat gga    355

Glu Pro Gly Ser Arg Tyr Gln Met His Ser Ser Lys Ser Ser     104
gaa cct gga agc aga tac cag atg cat agt agc aag tcc tct    397

Gly Arg Ser Gly Arg Ser Ser Tyr Lys Ser Lys His Arg Ser     118
ggt agg agt gga aga agc agt tac aaa agt aaa cac agg agt    439

Arg His His Thr Ser Gln His His Ser His Gly Lys Ser His    132
cgc cac cac aca tcg cag cac cat tca cac ggg aag agt cac    481

Arg Arg Lys Arg Ser Arg Ser Val Glu Asp Asp Glu Glu Gly    146
cga agg aaa aga tcg agg agt gta gag gat gat gag gag ggt    523

His Leu Ile Cys Gln Ser Gly Asp Val Leu Ser Ala Arg Tyr    160
cac ctg atc tgt cag agt gga gac gta cta agt gca aga tat    565

Glu Ile Val Asp Thr Leu Gly Glu Gly Ala Phe Gly Lys Val    174
gaa att gtt gat act tta ggt gaa ggt gct ttc gga aaa gtg    607

Val Glu Cys Ile Asp His Lys Val Gly Gly Arg Arg Val Ala    188
gtg gaa tgc atc gat cat aaa gtg gga ggt aga cgt gta gca    649

Val Lys Ile Val Lys Asn Val Asp Arg Tyr Cys Glu Ala Ala    202
gta aaa ata gtt aaa aat gtg gat aga tac tgt gaa gct gct    691

Gln Ser Glu Ile Gln Val Leu Glu His Leu Asn Thr Thr Asp    216
caa tcg gaa ata caa gtt ttg gaa cac ttg aat aca aca gac    733

Pro His Ser Thr Phe Arg Cys Val Gln Met Leu Glu Trp Phe    230
ccc cat agt act ttc cgt tgt gtc cag atg ttg gag tgg ttt    775

Glu His Arg Gly His Ile Cys Ile Val Phe Glu Leu Leu Gly    244
gag cat cga ggt cac att tgc att gtg ttt gaa ctt ctg ggg    817

Leu Ser Thr Tyr Asp Phe Ile Lys Glu Asn Ser Phe Leu Pro    258
ctt agt act tat gat ttc att aag gaa aac agt ttt ctg ccg    859

Phe Arg Met Asp His Ile Arg Lys Met Ala Tyr Gln Ile Cys    272
ttt cga atg gat cat atc agg aag atg gca tat caa ata tgc    901

Lys Ser Val Asn Phe Leu His Ser Asn Lys Leu Thr His Thr    286
aaa tct gta aac ttt ttg cat agt aat aaa ttg act cat aca    943

Asp Leu Lys Pro Glu Asn Ile Leu Phe Val Lys Ser Asp Tyr    300
gac ttg aag cct gaa aac atc tta ttt gtg aag tct gac tac    985

Thr Glu Ala Tyr Asn Pro Lys Met Lys Arg Asp Glu Arg Thr    314
aca gag gct tat aat ccc aaa atg aaa cgt gat gaa cgt act   1027

Ile Val Asn Pro Asp Ile Lys Val Val Asp Phe Gly Ser Ala    328
ata gta aat cca gat att aaa gtg gtg gac ttt gga agt gca   1069

Thr Tyr Asp Asp Glu His His Ser Thr Leu Val Ser Thr Arg    342
aca tat gat gat gaa cac cac agc aca ttg gta tct aca aga   1111

His Tyr Arg Ala Pro Glu Val Ile Leu Ala Leu Gly Trp Ser    356
cat tat aga gca ccg gaa gtt att tta gcc ctc ggg tgg tca   1153

Gln Pro Cys Asp Val Trp Ser Ile Gly Cys Ile Leu Ile Glu    370
cag cca tgt gat gtc tgg agc ata gga tgt att ctt atc gag   1195

Tyr Tyr Leu Gly Phe Thr Val Phe Ser Thr His Asp Ser Arg    384
tat tat ctt gga ttt aca gtt ttt tcg act cat gat agc agg   1237

Glu His Leu Ala Met Met Glu Arg Ile Leu Gly Pro Leu Pro    398
gaa cat tta gca atg atg gaa agg att ctt gga cca cta cca   1279

Lys His Met Ile Gln Lys Thr Arg Lys Arg Arg Tyr Phe His    412
aag cac atg ata cag aaa acc agg aaa cgc aga tat ttc cat   1321

His Asp Arg Leu Asp Trp Asp Glu His Ser Ser Ala Gly Arg    426
cat gat cga tta gat tgg gat gaa cac agt tct gct ggc aga   1363

Tyr Val Ser Arg Arg Cys Lys Pro Leu Lys Glu Phe Met Leu    440
tat gtt tct cgg cgc tgt aaa cct ctg aag gag ttt atg cta   1405

Ser Gln Asp Ala Glu His Glu Leu Leu Phe Asp Leu Ile Gly    454
tct cag gat gcc gaa cat gag ctt ctc ttt gac ctc att ggg   1447

Lys Met Leu Glu Tyr Asp Pro Ala Lys Arg Ile Thr Leu Lys    468
aaa atg ttg gag tat gat ccc gcc aaa aga att act ctc aaa   1489

Glu Ala Leu Lys His Pro Phe Phe Tyr Pro Leu Lys Lys His    482
gaa gcc cta aag cat cct ttc ttt tac cca ctt aaa aag cat   1531

Thr                                                       483
acgtgatttataaacacagtgctctgaaaggaatcttacagactgtatcagtcta  1586

gcttttaattaagttattttgtatagcttaatttgtaaaacattttatgttttt   1641

agatgctttattaaatacatggccaaaccaaataacatctttcagtaattataga  1696

atgatttatttgqaataaaatttgtgcttatgaatgtaaaaaaa             1741
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FIG. 2. STY cDNA and predicted amino acid sequence. The first ATG codon (position 85) is preceded by a stop codon in each reading frame and initiates an open reading frame of 1,449 nucleotides followed by a untranslated region and a poly(A) tail at the 3′ end of the molecule. The protein predicted by this open reading frame would have a molecular weight of 57 kDa and an isoelectric point of 8.9. The kinase domain is underlined, and the potential nuclear localization signal is boxed.

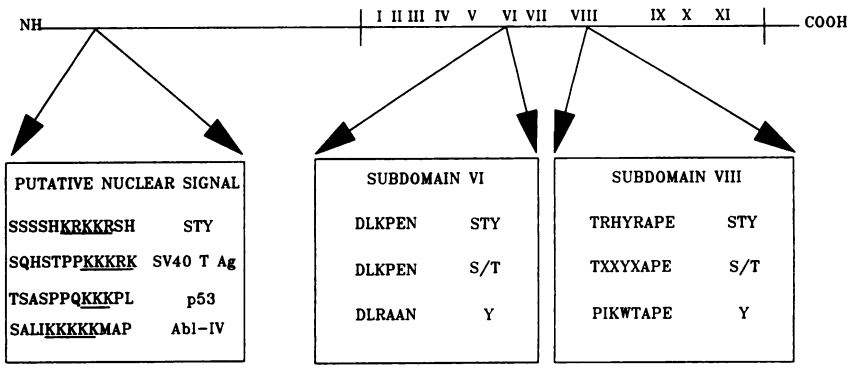

FIG. 3. Schematic representation of the STY kinase. Homologies to the kinase subdomain consensus sequence are indicated by roman numerals. Comparisons with nuclear localization signals or serine/threonine (S/T) and tyrosine (Y) kinase subdomains are shown in boxed areas. SV40 T Ag, simian virus 40 T antigen.

tyrosine kinase (Fig. 3; 1, 38). Several of the previously identified nuclear localization signals are flanked by hydroxy amino acids (Fig. 3), and in the case of p53 and simian virus 40 T antigen, these are targets for $p34^{cdc2}$ or an associated kinase in vitro (1, 26). The serine residues adjacent to the proposed nuclear localization signal of STY do not fit the consensus for $p34^{cdc2}$ phosphorylation (18) but may be recognized by another kinase.

The remainder of the STY protein contains the 11 subdomains which are thought to collectively make up the catalytic region of protein kinases (14). Although the STY cDNA was isolated with an antibody directed against phosphotyrosine, its catalytic domain shows greatest homology with the serine/threonine-type kinases when compared against the NBRF-PIR(r) data base using the Lipman-Pearson algorithm (22). The STY kinase shows 30% homology to the yeast *FUS3* gene, which is closely related to *cdc2/CDC28* (11). It also shows high homology to the second S6 kinase II domain (15). Notably, subdomains VI and VIII of STY, which are thought to be indicative of serine/threonine versus tyrosine specificity, match the serine/threonine consensus (Fig. 3). Similarly, a tryptophan residue found in subdomain XI of all tyrosine kinases (37) is not found in the STY kinase.

Despite this homology to serine/threonine kinases, the STY protein product had PTK activity, both as a full-length protein and as a fusion protein between the bacterial *trpE* gene and the STY catalytic domain (*trpE*-STY). Western blots on bacterial lysates from *trpE*-STY-expressing cells show that proteins migrating at 100 and 40 kDa were recognized by an antiphosphotyrosine antibody, and the 100-kDa protein reacted with an antibody directed against *trpE* (data not shown). These same two proteins were immunoprecipitated from bacterial lysates with either antibody, as evidenced by in vitro kinase assays performed on antiphosphotyrosine or anti-*trpE* immunocomplexes (Fig. 4A). The two phosphoproteins are closely related, as they share five phosphotryptic peptides (Fig. 4C). The 40-kDa protein is probably a proteolytic fragment of the 100-kDa fusion protein, with which it tightly associated during immunoprecipitation. The individually electroeluted phosphoproteins contained phosphoserine, phosphothreonine, and phosphotyrosine (Fig. 4B) whether the kinase assay was performed using the *trpE* or the phosphotyrosine antibody immune complexes (Fig. 4B). We were unable to dissociate the serine/threonine kinase activities form the tyrosine kinase activity either by washing the immune complexes with

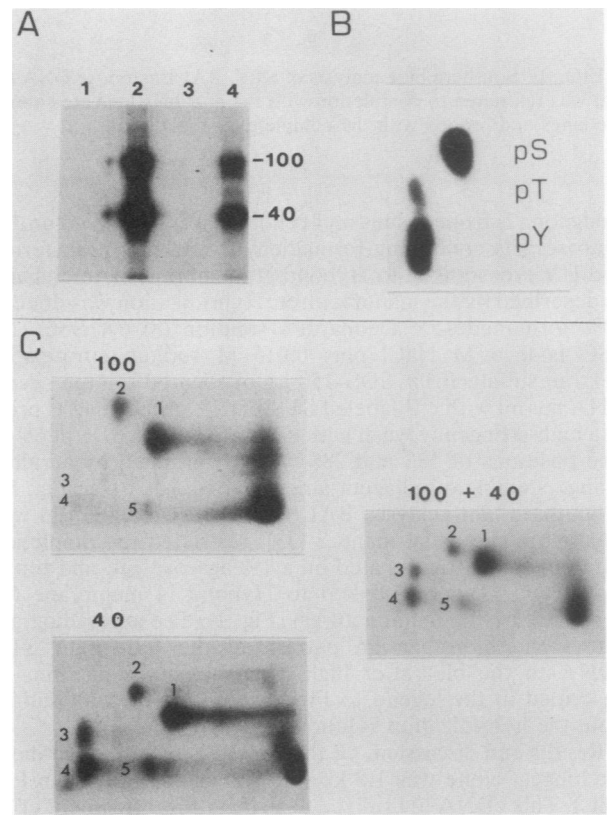

FIG. 4. In vitro kinase reactions of *trpE*-STY fusion protein in bacterial lysates. (A) Lysates of *E. coli* JM103 expressing either the *trpE* protein (lanes 1 and 3) or the *trpE*-STY fusion protein (lanes 2 and 4) were immunoprecipitated with either antibody for phosphotyrosine (PY 20) (lanes 1 and 2) or *trpE* AB-1 (monoclonal antibody for *trpE* from Oncogene Science Inc.) (lanes 3 and 4) and assayed for kinase activity with [γ-^{32}P]ATP prior to SDS-polyacrylamide gel electrophoresis. (B) Phosphoamino acid analysis of electroeluted products from panel A. The positions of phosphoamino acid standards (pT, pS, and pY for phosphothreonine, phosphoserine, and phosphotyrosine, respectively) are indicated beside the autoradiogram. (C) Phosphotryptic maps of the 100-kDa, 40-kDa, and mixed 100- and 40-kDa electroeluted proteins. Numbers 1 through 5 represent shared phosphopeptides.

0.5 M NaCl or by specifically eluting the 100- and 40-kDa phosphoproteins from the antiphosphotyrosine immune complexes with phenylphosphate prior to the initiation of the kinase reaction. *E. coli* has no detectable PTKs (see reference 21 and references within); therefore the tyrosine kinase activity in immunoprecipitates must be an endogenous property of the STY expression product. While it is possible that the serine/threonine kinase activity resulted from a copurifying bacterial enzyme, the sequence homologies of STY (Fig. 3) suggest that this activity is intrinsic to STY.

The expression of STY is developmentally regulated at the transcript level. In P19 stem cells, a single mRNA species of 1.8 kb was detected. Treatment of P19 cell aggregates with RA initiated their differentiation into neurons and astroglia, whereas DMSO treatment produced cardiac and skeletal muscle cells. Concomitant with differentiation into either lineage, two additional STY transcripts were detected (3.2 and 5.6 kb). In RA-treated cultures, the ratios of the three forms remained essentially constant up to day 7, while there was an overall increase in the level of expression compared with the level of standardization (Fig. 5A). In contrast, with DMSO treatment, the expression of all three forms decreased with time (Fig. 5B). Once the blot shown in Fig. 5B was normalized to the expression of β-actin, we detected a fivefold decrease in the expression of STY by day 7. When the STY cDNA was used to probe a Northern blot of adult tissues, all three major transcripts were detected, although their relative abundances varied among tissues (Fig. 5C). It is of interest to note that, like the P19 stem cell, SP10, a myeloma cell line, and P388, a pre-B-cell leukemia line, expressed only the 1.8-kb transcript.

In other systems, alternative splicing of primary transcripts or differential promoter activity is responsible for the generation of multiple transcripts from single-copy kinase genes (5, 19, 24, 29, 38, 39). These additional mRNA species encode proteins with variations in the domains necessary for subcellular distribution, ligand binding, or catalytic activity. The developmental regulation of the expression of STY transcripts suggests that multiple isoforms of this kinase may also exist.

We report here the identification of STY, a novel developmentally regulated kinase. It has highest homology to serine/threonine consensus elements, and yet immunoprecipitates of bacterially expressed STY phosphorylate tyrosine as well as serine and threonine. This suggests that STY belongs to a previously unappreciated family of kinases which have the potential to phosphorylate all three hydroxy amino acids. In support of this we have recently identified two additional cDNAs which share homology to serine/threonine subdomains VI and VIII and yet were cloned on the basis of their reactivity to the antiphosphotyrosine antibody (unpublished data). We are presently generating antibodies to bacterially expressed STY protein in order to assess its subcellular localization and biochemical activity in mammalian cells.

GenBank accession number. The GenBank accession number for the STY kinase sequence is M38381.

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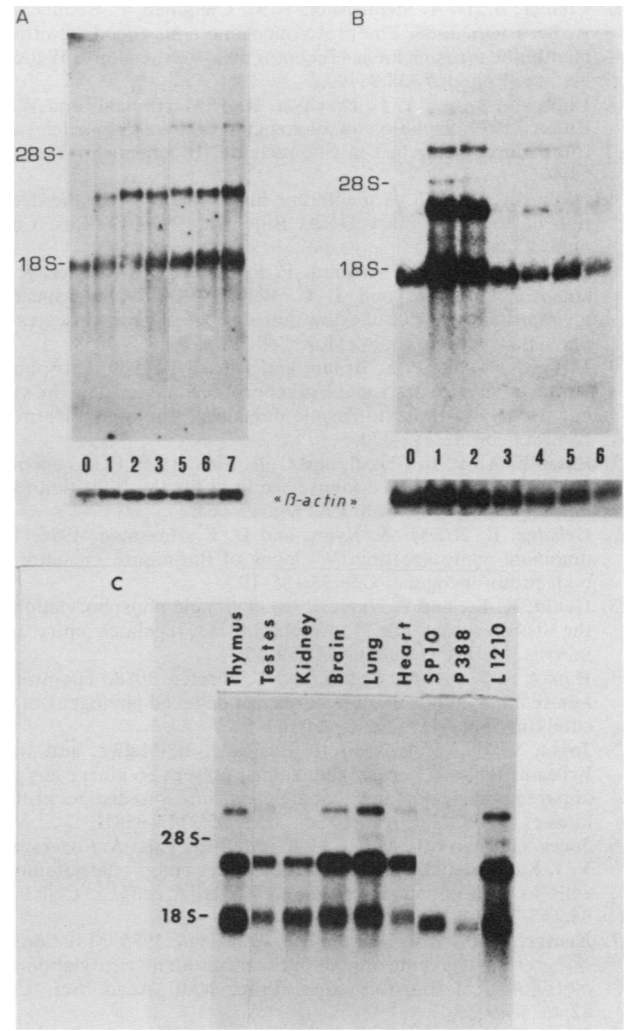

FIG. 5. Developmental expression of STY transcripts. Each lane represents the hybridization of the complete STY cDNA to poly(A)$^+$ RNA isolated from P19 EC stem cells as described elsewhere (2) (A and B, lanes 0) and their differentiated derivatives at days indicated by lane numbers during either RA (A) or DMSO (B) differentiation protocols (16, 25). Radiograms at the bottom of each panel show the comparative hybridization of β-actin cDNA to each lane. (C) Hybridization of STY to RNA from adult tissues and leukemic cell lines as indicated above each lane.

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