The EMBO Journal vol.10 no.2 pp.317-325, 1991

A mammalian protein kinase with potential for serine/threonine and tyrosine phosphorylation is related to cell cycle regulators

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Communicated by J.Schlessinger

In a screen of mouse erythroleukemia cDNA expression libraries with anti-phosphotyrosine antibodies, designed to isolate tyrosine kinase coding sequences, we identified several cDNAs encoding proteins identical or very similar to known protein-tyrosine kinases. However, two frequently isolated cDNAs, *clk* and *nek*, encode proteins which are most closely related to protein kinases involved in regulating progression through the cell cycle, and contain motifs generally considered diagnostic of protein-serine/threonine kinases. The *clk* gene product contains a C-terminal *cdc2*-like kinase domain, most similar to the *FUS3* catalytic domain. The Clk protein, expressed in bacteria, becomes efficiently phosphorylated *in vitro* on tyrosine as well as serine/threonine, and phosphorylates the exogenous substrate poly(glu, tyr) on tyrosine. Direct biochemical evidence indicates that both protein-tyrosine and protein-serine/threonine kinase activities are intrinsic to the Clk catalytic domain. These results suggest the existence of a novel class of protein kinases, with an unusual substrate specificity, which may be involved in cell cycle control.

Key words: anti-phosphotyrosine antibodies/protein phosphorylation

Introduction

Protein kinases regulate a wide array of cellular responses to changing environmental conditions. In eukaryotes, all described protein kinases are specific for phosphorylation of the hydroxyamino acids serine and threonine or tyrosine (Hunter and Cooper, 1985; Edelman *et al.*, 1987; Hanks *et al.*, 1988), although it is likely that eukaryotic protein kinases capable of phosphorylating histidine, arginine and lysine also exist (Wold, 1981). Phosphorylation is of particular significance in controlling mitogenesis and cellular differentiation. Receptors for a number of polypeptide growth factors are transmembrane tyrosine kinases (Yarden and Ullrich, 1988), which in turn stimulate serine/threonine kinases such as protein kinase C, MAP kinase and $p74^{raf}$ (Hunter *et al.*, 1984; Rossomondo *et al.*, 1989; Morrison *et al.*, 1989).

The eukaryotic cell cycle is controlled by the $p34^{cdc2}$ serine/threonine kinase, whose activity is modified through the cell cycle by phosphorylation and interactions with cyclins (reviewed in Lewin, 1990). $p34^{cdc2}$ is enzymatically inactivated by phosphorylation on tyrosine, which thereby inhibits entry into mitosis (Gould and Nurse, 1989; Morla *et al.*, 1989). The protein kinases which phosphorylate $p34^{cdc2}$ have not been biochemically characterized, although the *wee1* gene, which negatively regulates *cdc2*, encodes a potential protein kinase with sequence motifs generally considered diagnostic of protein-serine/threonine kinases (Russell and Nurse, 1987). Thus far no classical tyrosine kinases have been cloned from yeast.

Despite the evident interplay between serine/threonine- and tyrosine-specific protein kinases, previously identified eukaryotic protein kinases have shown a strict specificity for either serine/threonine or tyrosine (see Weinmaster and Pawson, 1986; Moran *et al.*, 1988). A comparison of protein kinase catalytic sequences has revealed two types of conserved residue—amino acids found in all protein kinases, which probably are critical in folding or catalysis, and amino acids conserved among tyrosine kinases but not serine kinases, and *vice versa* (Hanks *et al.*, 1988). These latter are good candidates for residues involved in substrate specificity. For example the sequence HRDLXXXN is relatively well conserved in all protein kinases (Hanks *et al.*, 1988) and the invariant aspartate is essential for kinase activity (Moran *et al.*, 1988; Tan *et al.*, 1990). In protein-tyrosine kinases the variable (X) residues are AAR or RAA, whereas in protein serine/threonine kinases they are most frequently KPE, with the lysine being invariant. Similar arguments can be made for the sequence immediately N-terminal of the conserved residues APE in the protein kinase subdomain VIII (Hanks *et al.*, 1988).

cDNAs for many novel protein kinases have been cloned using degenerate oligonucleotide probes, PCR techniques or hybridization to an existing cDNA at low stringency (Hanks *et al.*, 1988; Wilks, 1989; Hirai *et al.*, 1987). We and others have employed a functional screen for tyrosine kinase cDNAs in which affinity-purified anti-phosphotyrosine (P.Tyr) antibodies are used to probe a cDNA expression library (Kornbluth *et al.*, 1988; Letwin *et al.*, 1988; Lindberg *et al.*, 1988; Pasquale and Singer, 1989). A principal attraction of this approach is that it does not depend on recognition by a tyrosine kinase-specific oligonucleotide probe, and therefore should allow the cloning of cDNAs that do not conform to the protein-tyrosine kinase consensus sequence. Here we describe the isolation of cDNAs from mouse erythroleukemia cell cDNA expression libraries using anti-P.Tyr antibodies. Two of the most frequently isolated cDNAs encode proteins most closely related to the *cdc2* and *nimA* cell cycle protein kinases, and contain motifs diagnostic of protein-serine/threonine kinases. However, we show biochemically that the *cdc2*-like kinase catalytic domain can efficiently phosphorylate tyrosine as well as serine/threonine.

Results

Isolation of protein-tyrosine kinase cDNAs from Friend mouse erythroleukemia cell expression libraries using anti-P.Tyr antibodies

The mRNAs from two Friend erythroleukemia cell lines were reverse transcribed, and cDNA expression libraries were constructed in λgt11. Friend cells correspond phenotypically to immature hematopoietic cells of the erythroid lineage, and retain a capacity for erythroid differentiation after treatment with one of a number of chemical inducers, such as DMSO (Friend *et al.*, 1971). Protein-tyrosine kinases expressed in these cells might be involved in regulating erythroid development or cellular proliferation. We therefore screened the unamplified λgt11 libraries with anti-P. Tyr antibodies, a procedure which yielded a total of 20 positive clones from 2×10^6 phage examined. These cDNA clones, which based on antibody screening are likely to encode protein-tyrosine kinases, were partially or completely sequenced. In some instances the identity of a specific clone was established by cross-hybridization with other isolates. The results of this analysis are summarized in Table I.

Several clones were identified as being identical or closely related to previously isolated protein-tyrosine kinases. Three of the clones contained murine *fer* transcripts and one of these, clone F2, contained a full-length *fer* cDNA (Letwin *et al.*, 1988; Hao *et al.*, 1989). Clone C2 encoded a partial *lyn* message (Yamanishi *et al.*, 1987). Clone Q1 contained a partial cDNA whose expected translation product is very similar to the recently identified rat *elk* PTK (Letwin *et al.*, 1988). The relationship between the two sequences would suggest that Q1 is an *elk*-related gene rather than the murine *elk* equivalent (J.McGlade and V.Lhotak, unpublished results). The insert in clone E2 was apparently derived from mouse *flg*, which encodes a fibroblast growth factor-receptor (Ruta *et al.*, 1988, 1989; Lee *et al.*, 1989).

Identification of two novel protein kinases clk and nek, related to cdc2 and nimA

The remaining clones were categorized into three groups (Table I). The sequence of the entire 1661 nucleotide insert of the E3 cDNA was determined and found to contain a single long open reading frame of 1519 nucleotides extending from the extreme 5′ end of the clone. Additional 3′ sequences were derived from the overlapping R1 cDNA (Figure 1). These cDNAs are probably derived from a 1.8 kb mRNA (see below), indicating that the composite sequence is nearly full-length. The translation product initiating at the 5′-most AUG codon would be 483 amino acids in length with an expected molecular weight of 57 kd, although we have not yet ruled out the possibility that the E3 cDNA lacks some 5′ coding sequence. *In vitro* transcription and translation of the E3 cDNA yielded a protein with an apparent molecular weight of 55 kd in a rabbit reticulocyte lysate, in agreement with the size predicted from sequence analysis (data not shown). Analysis of the expected translation product of the E3 clone revealed the presence of all the characteristic protein kinase catalytic domain sequence motifs. Surprisingly, the expected E3 protein contains those sequences which would distinguish it as a serine/threonine-specific rather than a tyrosine-specific protein kinase. A search of GenBank and EMBL databases confirmed E3 as a novel sequence, although it was most similar to members of the

Table I. Identities of clones isolated from mouse erythroleukemia cell λgt11 cDNA libraries by screening with anti-phosphotyrosine antibodies

λgt11 clones	Identity
F1,F2,H3	*fer*
C2	*lyn*
Q1	*elk*-related
E2	*flg* (FGF-R)
A1,A2,B1,H1,H4,J1,J2,N2,N3,P1	*nek*
C1,E3,R1	*clk*
S1	?

Clones were identified by direct sequence analysis and, in some cases, by cross-hybridization. Since the library was not amplified, each isolate represents a unique cDNA.

CDC28/cdc2$^+$ family of protein kinases. Most of the kinases comprising this family have been identified in yeast and are thought to govern critical decision points in the yeast cell cycle. For this reason, we have designated the E3 clone as *clk* (pronounced 'clik'), for *C*DC28/cdc2$^+$ -*l*ike *k*inase. The *clk* product is ~34% identical to *FUS3* (Elion *et al.*, 1990), 32% identical to YAK1 (Garrett and Broach, 1989), 30% identical to cdc2Hs (Lee and Nurse, 1987) and MAP kinase/*ERK1* (Boulton *et al.*, 1990), 28% identical to CDC28 (Lorincz and Reed, 1984), and 27% to KSS1 (Courchesne *et al.*, 1989) over their kinase domains (Figure 2). Particularly striking is a short region of strong homology shared by all the CDC28/cdc2$^+$ family of kinases, corresponding to residues 451–476 of Clk, situated in the extreme carboxy terminus of the catalytic domain in a region which is otherwise not well conserved among most other protein kinases.

From the group comprising the most frequently isolated cDNA, the entire nucleotide sequence of the N2 cDNA insert was determined. The expected N2 translation product contains all the sequences characteristic of protein kinase catalytic domains (K.Letwin, Y.Ben-David and T.Pawson, manuscript in preparation). Like *clk*, N2 contains residues which are conserved among the serine/threonine-specific protein kinases and lacks those residues generally associated with the tyrosine-specific protein kinases. A search of the GenBank and EMBL databases with the N2 sequence revealed it to be a novel protein, although it showed the highest similarity to known protein kinases. The greatest similarity was to the *nimA* protein, a putative serine/threonine kinase involved in regulating the G_2–M phase transition in the fungus *Aspergillus nidulans* (Morris, 1976; Osmani *et al.*, 1988). Both proteins were ~30% identical overall and 43% identical across their catalytic domains. Based on its similarity to N*imA*, we have designated the N2 clone as *nek* for *n*imA-*r*elated *k*inase.

The final clone, S1, does not fall into any of the previously identified groups. Partial sequence analysis indicates that the S1 cDNA product contains a novel protein kinase catalytic domain with several hallmarks of protein-serine/threonine kinases (L.Tannock, unpublished results). Hence all of the cDNA clones identified in the screen encode polypeptides with sequences characteristic of protein kinase domains.

Protein kinase activity of a bacterially expressed TrpE–Clk fusion protein

The repeated isolation of *clk* and *nek* cDNAs in a screen employing anti-P.Tyr antibodies is paradoxical, since the

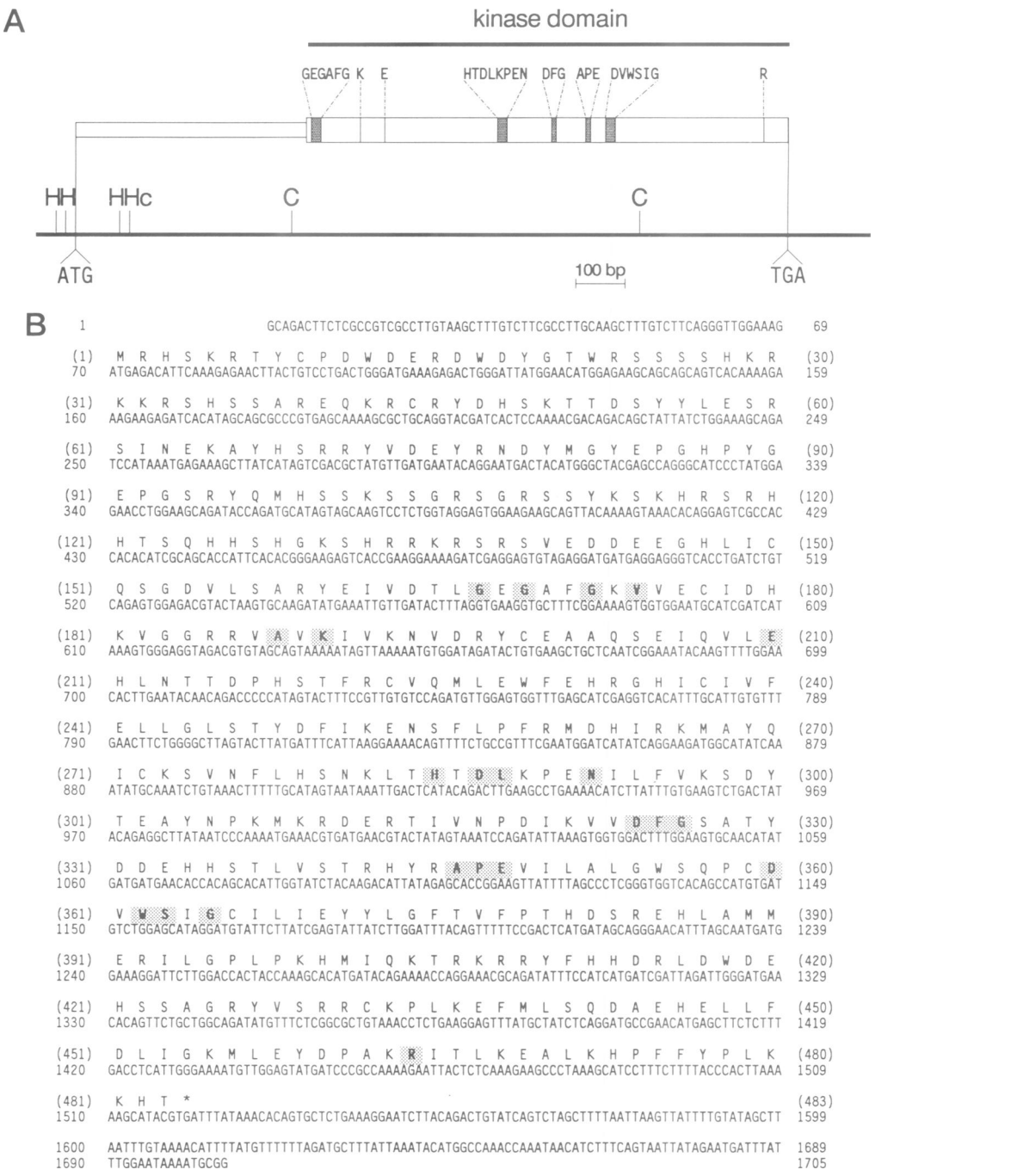

Fig. 1. Sequence and structure of the *clk* cDNA and its primary translation product. **A**. The 1.7 kb *clk* cDNA is shown with the presumed initiation and termination codons indicated. Restriction endonuclease sites are also shown (H, *Hin*dIII; Hc, *Hin*cII; C, *Cla*I). The expected protein product is depicted above the cDNA, with the catalytic domain and the positions of several highly conserved seqence motifs highlighted. **B**. Sequence of the *clk* cDNA and its primary translation product. Conserved residues diagnostic of a protein kinase catalytic domain are highlighted. The single letter amino acid designation is used.

cDNAs encode proteins that would normally be classified as protein-serine/threonine kinases on the basis of their primary structures. There are two possible explanations for these observations. The anti-P.Tyr antibodies might recognize epitopes created by the expression of *clk* or *nek* in bacteria which do not, in fact, contain P.Tyr. Alternatively, *clk* and *nek* may possess tyrosine kinase activity.

To confirm the protein kinase activity of Clk and determine its hydroxyamino acid specificity, a portion of the E3 clone containing all but the extreme 5′ end of the *clk* cDNA was subcloned into a pATH bacterial expression vector in order to generate a TrpE−Clk fusion protein. This protein contains Clk residues 79−483, including most of the N-terminal region and the entire presumptive catalytic domain. Immunoblots of lysates from cells expressing TrpE−Clk using anti-P.Tyr antibody showed two major bands of ~85 kd, corresponding to the expected size of the TrpE−Clk fusion protein, and 45 kd (Figure 3). No signal was seen in lysates of cells expressing TrpE alone. When induced bacterial lysates were immunoprecipitated with anti-P.Tyr antibodies, and the immunoprecipitates analyzed by western blotting with anti-TrpE antibodies, the same 85 kd

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Clk    157  SARYEIVDTLGEGAFGKVVECIDHKV--GGRRVAVKIVKNVDRYC---EAAQSEIQVLEHLN-TTDPHSTFRCVQMLEWFEHRGHICIVFELLGLSTYDF  250
FUS3    10  .SDFQLKSL.....Y.V.CSATHKP---T.EI..I.KIEPF.KPLFA-LRTLR..KI.K.F--KHENII.IFNI.RPDS..NFNEVY.IQ..MQTDLHRV  103
YAK1   366  NRK.LVL.I..Q.T..Q..K.QNLL---TKEIL...V..SRTE.L---TQSIT.AKI..L..QKI..TNKHHFLR.YDS.V.KN.L.L.....SNNL.EL  459
ERK1    27  GP..TQLQYI....Y.M.SSAY..V---RKT...I.KISPFEHQTYC-QRTLR...I.LGF--RHENVIGI.DILRAPTL.AMRDVY..QD.METDL.KL  120
CDC2Hs   1  MED.TKIEKI...TY.V.YKGRHKT---T.QV..M.KIRLESEEEGVPST.IR..SL.KE.--RHPNIVSLQD.L.QD-----SRLYLI..F.SMDLKKY   90
CDC28    5  L.N.KRLEKV...TY.V.YKAL.LRPGQ.Q.V..L.KIRLESEDEGVPST.IR..SL.KE.--KD.NIVRLYDIVHSDA----HKLYL...F.D.DLKRY   98

Clk    251  IKENSFLP-FRMDHIRKMAYQICKSVNFLHSNKLTHTDLKPENILFVKSDYTEAYNPKMKRDERTIVNPDIKVVDFGSATYDDEHH-----------S  336
FUS3   104  .STQM----LSD...QYFI..TLRA.KV..GSNVI.R....S.L.INSNC-------------------L..C...L.RII..SAADNSEPTGQQSGMT  180
YAK1   460  L.Q.K.HG-LSIQL..TFTT..LD.LCV.KES..I.C.......LCAP.KP----------------EL.II....SCEEARTV-------------Y  528
ERK1   121  L.SQQ----LSN...CYFL...LRGLKYI..ANVL.R....S.L.INTTC-------------------L.IC...L.RIA.PE.DHTGFL-------T  190
CDC2Hs  91  LDSIPPGQYMDSSLVKSYL...LQGIV.C..RRVL.R....Q.L.IDDKG------------------T..LA...L.RAFGIPIRVY----------T  161
CDC28   99  MEGIPKDQPLGA.IVK.FMM.L..GIAYC..HRIL.R....Q.L.IN.DG------------------NL.LG...L.RAFGVPLRAY----------T  169

Clk    337  TLVSTRHYRAPEVI-LALGWSQPCDVWSIGCILIEYYLGFTVFPTHDSREHLAMMERILGPLPKHMIQKTRKRRYFH----HDRLDWDEHSSAGRYVSRR  431
FUS3   181  EY.A..W......MLTSAKY.RAM....C....A.LF.RRPI..GR.Y.HQ.LLIFG.I.TPHSD---------------N.LRCIESPRAREYIK.LP  264
YAK1   529  .YIQS.F.....I.-.GIPY.TSI.M..L...VA.LF..IPI..GASEYNQ.TRIIDT..YP.SW..DMGKNSGK.M(36)YFKWRKLPDIIRNYRYPKS  659
ERK1   191  EY.A..W.....IMLNSK.YTKSI.I..V....A.MLSNRPI..GKHYLDQ.NHILG...SPSQ----------------E.LNCIINMKARNYLQ.LP  273
CDC2Hs 162  HE.V.LW..S...LLGSARY.T.V.I....T.FA.LATKKPL.HGDSEIDQ.FRIF.A..TP-----------------NNEVWPEVE.LQDYKNTFP  242
CDC28  170  HEIV.LW......LLGGKQY.TGV.T......FA.MCNRKPI.SGDSEIDQIFKIF.V..TP-----------------NEAIWP.IVYLPDFKP.FP  250

Clk    432  CKPLKEFMLSQDAEHEFLFDLVGKILEYDPAKRITLKEALKHPFFYPLKKHT  484
FUS3   265  MY.AAPLEKMFPRVNPKGI..LQRM.VF.....A....E..YLQTYHDPN  316
YAK1   660  IQNSQ.LIDQEMQNR.C.IHFL.GV.NLN.LE.W.PQQ.ML...ITKQEFTG  711
ERK1   274  S.TKVAWAKLFPKSDSKAL..LDRM.TFN.N....VE...A..YLEQYYDP.  325
CDC2Hs 243  KWKPGSLASHVKNLD.NGL..LS.M.I.....SG.M..N..Y.ND.DNQI  294
CDC28  251  QWRR.DLSQVVPSLDPRGI..LD.L.A...IN..SARR.AI..Y.QES      298
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Fig. 2. Comparison of the catalytic domains of Clk and other members of the *cdc2*$^+$ family. Residues identical to those in Clk are indicated by dots. Gaps are indicated by hyphens. Highly conserved protein kinase residues are shaded.

and 45 kd polypeptides were detected (data not shown). This confirmed the identity of the tyrosine phosphorylated 85 kd protein as the TrpE−Clk fusion protein, and suggested the P.Tyr-containing 45 kd protein to be a breakdown product of the 85 kd protein which contains N-terminal TrpE determinants. If the 45 kd polypeptide contains the entire 37 kd TrpE sequence, it would be expected to have most of the N-terminal Clk region but little, if any, of the catalytic domain. The signal seen in anti-P.Tyr immunoblots of TrpE−Clk lysates was completely blocked by preincubating the anti-P.Tyr antibody with 5 mM P.Tyr, while no detectable reduction in signal was observed by preincubation with either 5 mM phosphoserine or 5 mM phosphothreonine (data not shown).

We next tested the kinase activity of the TrpE−Clk protein in an *in vitro* assay. Immunoprecipitates were prepared from bacterial cells expressing either TrpE−Clk or TrpE alone using control, anti-TrpE or anti-P.Tyr antibodies. The immune complexes were then incubated with [γ-^{32}P]ATP. Kinase reactions containing anti-TrpE or anti-P.Tyr immunoprecipitates of TrpE−Clk cell lysates showed two major ^{32}P-containing proteins of apparent molecular weights 85 kd and 45 kd (Figure 4A). These correspond in size to the full-length TrpE−Clk fusion product, and the major breakdown product identified by immunoblotting. The *in vitro* phosphorylation of these proteins was specific, and was not observed in any control reactions. Phosphoamino acid analysis of the *in vitro* phosphorylated 85 kd and 45 kd proteins, immunoprecipitated with either anti-trpE or anti-P.Tyr antibodies, showed roughly equivalent amounts of both phosphoserine and phosphotyrosine as well as small amounts of phosphothreonine (Figure 4B).

These results suggested that the Clk catalytic domain might have the potential to autophosphorylate on both serine/threonine and tyrosine. However an alternative interpretation would be that one or other of these kinase activities is supplied by a contaminating bacterial protein. To investigate this point, we altered the codon for lysine-190 to arginine by site-directed mutagenesis of the *clk* cDNA. Lysine-190 corresponds to a residue which is invariant in all protein kinases and is critical for kinase activity, probably because it interacts with the γ-phosphate of ATP at the kinase active site (Weinmaster and Pawson, 1986; Hanks *et al.*, 1988). An 85 kd TrpE−Clk protein with arginine at residue 190 was defective for both tyrosine and serine/threonine phosphorylation *in vitro* (data not shown), suggesting that both these activities are intrinsic to the Clk kinase domain. In an alternative approach, we used a procedure in which proteins are separated by gel electrophoresis, transferred to a filter, renatured and incubated with [γ-^{32}P]ATP (Ferrell and Martin 1989, 1990). In this assay, protein kinases are detected by their ability to autophosphorylate when immobilized on a filter. For this purpose, whole cell lysates from bacteria expressing TrpE or TrpE−Clk were resolved on an SDS−polyacrylamide gel and transferred to a poly vinylidene difluoride (PVDF) membrane. The blotted proteins were then treated with guanidinium chloride, allowed to renature overnight, and then overlayed with [γ-^{32}P]ATP in an *in vitro* kinase reaction. A single ^{32}P-labelled protein was seen in the lysate of TrpE−Clk expressing cells, which migrated at an apparent molecular weight of 85 kd (Figure 5A). Phosphoamino acid analysis of the 85 kd band yielded equivalent amounts of phosphoserine and phosphotyrosine, with a lower amount of phosphothreonine (Figure 5B). The simplest explanation

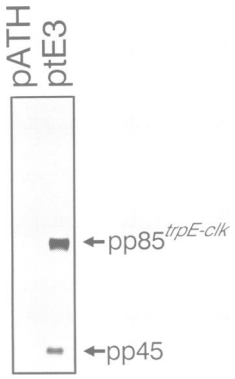

Fig. 3. A bacterially expressed TrpE−Clk protein is phosphorylated on tyrosine. Lysates from bacteria induced to express TrpE alone (pATH) or a TrpE−Clk protein (ptE3) were resolved by SDS−PAGE, transferred to nitrocellulose and immunoblotted with antiphosphotyrosine antibodies. The 85 kd TrpE−Clk protein and the 45 kd protein are indicated.

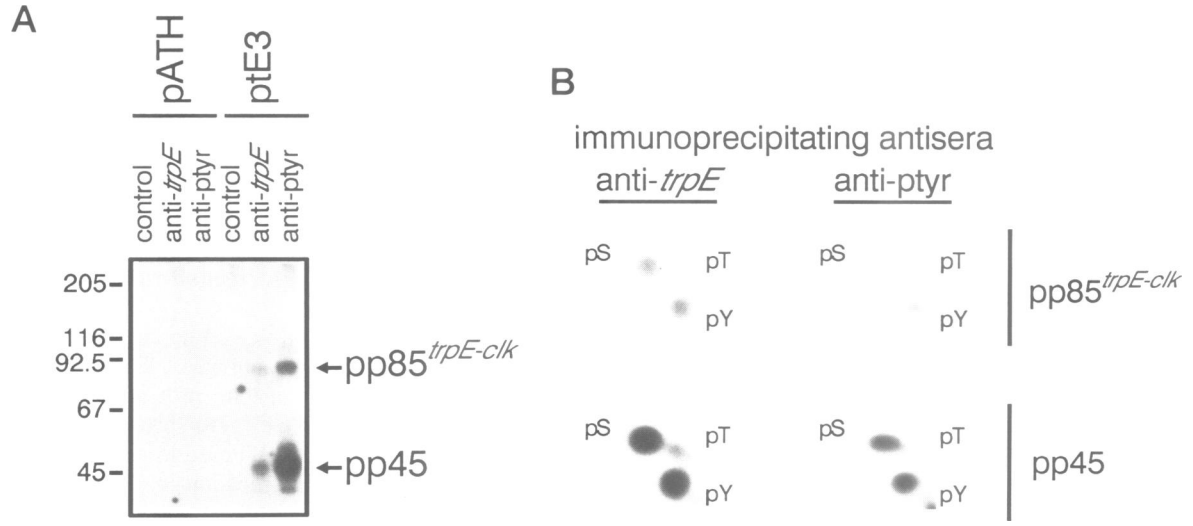

Fig. 4. A. *In vitro* kinase activity of a bacterially expressed TrpE−Clk protein. Lysates from TrpE−Clk expressing cells (ptE3) or cells expressing TrpE alone (pATH) were immunoprecipitated with either control, anti-TrpE or anti-P.Tyr antibodies, using heat inactivated *S.aureus*. Immunoprecipitates were incubated with [γ-^{32}P]ATP and the reaction products separated by SDS−PAGE and exposed to X-ray film for 18 h. The molecular weights ($\times 10^{-3}$) of protein markers are indicated. **B.** Phosphoamino acid analysis of the *in vitro* phosphorylated TrpE−Clk protein. TrpE−Clk proteins were immunoprecipitated with either anti-TrpE or anti-P.Tyr antibodies, and labeled in an *in vitro* kinase reaction. ^{32}P-labeled 85 kd and 45 kd bands were isolated, hydrolyzed to free phosphoamino acids, resolved by 2-dimensional electrophoresis and exposed to X-ray film for 7 days using an intensifying screen. pS = phosphoserine; pT = phosphothreonine; pY = phosphotyrosine.

for these results is that the 85 kd TrpE−Clk protein is able to phosphorylate all three hydroxyamino acids when immobilized on a filter. An alternative interpretation would be that a co-migrating bacterial protein kinase is able to transphosphorylate specifically the TrpE−Clk protein, although this seems unlikely. The 45 kd protein identified by anti-P.Tyr and anti-TrpE immunoblots, and in the immune complex kinase reaction, was not detected in the filter kinase assay. Since this 45 kd polypeptide contains TrpE sequences it is unlikely to contain an intact kinase domain, and would therefore not be expected to autophosphorylate. An implication of these results is that the phosphorylation of the 45 kd polypeptide in the immune complex kinase reaction may occur in *trans*, with the 45 kd protein acting as a substrate for the active 85 kd kinase. Alternatively, the 45 kd fragment may arise by proteolysis of the 85 kd fragment after autophosphorylation.

If Clk is a protein-tyrosine kinase, it should be able to phosphorylate an exogenous substrate on tyrosine. Consistent with this possibility, TrpE−Clk phosphorylated poly(glu,tyr) in an *in vitro* kinase reaction (Figure 6), illustrating that Clk has protein-tyrosine kinase activity despite its structural similarity to protein-serine/threonine kinases.

The clk gene is widely expressed and conserved in evolution

To examine the expression of *clk*, we carried out a Northern blot analysis of RNAs isolated from a variety of malignant cell lines and different mouse tissues. Under stringent hybridization conditions, the *clk* cDNA probe identified two widely expressed transcripts of 3.3 kb and 1.8 kb. In all the tissues that we analyzed, including spleen and thymus, the 3.3 kb transcript was the predominant *clk* RNA species (Figure 7). In contrast, a variety of malignant lymphoid and erythroid cell lines all contained a major 1.8 kb *clk* RNA and a minor 3.3 kb species (data not shown). Poly(A)$^+$ RNA prepared from two mouse erythroleukemia cell lines,

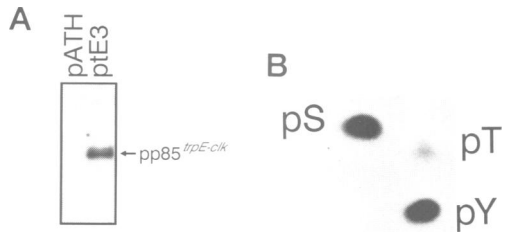

Fig. 5. TrpE−Clk phosphorylates on serine, theronine and tyrosine in a filter renaturation assay. **A.** Autophosphorylation of an immobilized TrpE−Clk protein in a denaturation/renaturation kinase assay. Lysates from 500 μl of TrpE−Clk expressing bacteria (ptE3) or TrpE expressing bacteria (pATH) were resolved by SDS−PAGE and transferred to a PVDF membrane. Blotted proteins were denatured in a guanidinium chloride solution and allowed to renature overnight at 4°C. The blot was overlayed with kinase reaction buffer containing [γ-^{32}P]ATP for 30 min, extensively washed and exposed to X-ray film for 2 h. **B.** Phosphoamino acid analysis of the autophosphorylated 85 kd TrpE−Clk protein from a denaturation/renaturation kinase assay. The PVDF membrane fragment containing the autophosphorylated protein was immersed in 6 N HCl. Phosphoamino acids were subsequently resolved by 2-dimensional electrophoresis prior to autoradiography for 18 h.

TP3 and CB7, also contained predominantly the 1.8 kb transcript (data not shown). Hence, there appears to be differential regulation of *clk* expression, with the 1.8 kb transcript being favored in rapidly proliferating tumour cells.

These two RNA species may be derived by differential processing of a *clk* transcript, or might represent transcripts from two distinct genes. Southern blot analysis of DBA mouse genomic DNA digested with the restriction enzyme *Bgl*II, using a *clk* cDNA probe, identified a single 17 kb band (data not shown). This suggests that *clk* is a single copy gene, and that the 1.8 kb and 3.3 kb RNAs may be generated by differential initiation, termination or splicing of the *clk* transcript. Since the 1.8 kb *clk* transcript is the predominant species in the erythroleukemia cells from which the

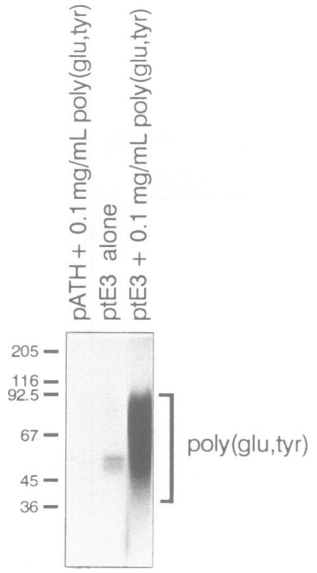

Fig. 6. TrpE−Clk phosphorylates poly (glu, tyr) *in vitro*. Antiphosphotyrosine immunoprecipitates of TrpE−Clk (ptE3) or TrpE alone (pATH) were incubated in a kinase reaction buffer containing [γ-^{32}P]ATP or [γ-^{32}P]ATP plus poly(glu, tyr) 4:1 at a final concentration of 100 μg/ml. Reaction products were resolved by SDS−PAGE and exposed to X-ray film for 2 h. The molecular weights ($\times 10^{-3}$) of protein markers are indicated.

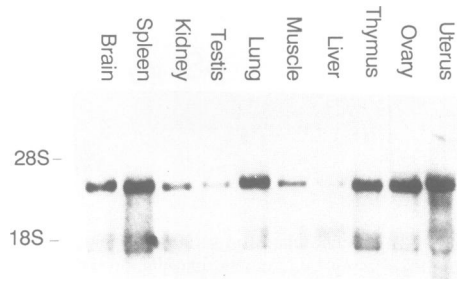

Fig. 7. Expression of *clk* transcripts in mouse tissues. Total cellular RNA (20 μg) from different mouse tissues was denaturated with formaldehyde, electrophoresed in an agarose gel, blotted onto nitrocellulose paper and hybridized with a *clk* cDNA probe. 28S RNA and 18S RNA have sizes of 4.4 kb and 2 kb respectively.

λgt11 libraries were made, it seemed likely that the 1.7 kb *clk* cDNA insert corresponds to this smaller mRNA. Rescreening of the erythroleukemia cDNA library resulted in the repeated isolation of cDNAs of 1.6 kb or smaller with an identical restriction map to the E3 *clk* insert.

Since *clk* is related to genes found in fungi, we analyzed *Hin*dIII-digested genomic DNA from a variety by species of Southern blotting with a *clk* probe, using moderately stringent hybridization conditions. The *clk* cDNA probe detected bands from the genomic DNAs of man, mouse, rat, chicken and *Drosophila melanogaster* (data not shown). These results suggest that the *clk* gene has been highly conserved during evolution. The chromosomal location of the mouse *clk* locus was determined by following the distribution of polymorphic alleles of the *clk* gene in recombinant inbred (RI) mouse strains derived from matings between C57BL/6J and DBA/25 mice. Analysis of DNA from 26 RI mice revealed a 100% co-segregation pattern between *clk* and the aldehyde oxidase (AOX) locus localized on mouse chromosome 1 (data not shown). These results place *clk* within 2 cM of the AOX locus, and, as no other protein kinase gene has been reported to map on this region, provide further evidence that it is a novel, single copy gene.

Discussion

A mammalian protein kinase related to members of the cdc2 gene family

In a screen of mouse erythroleukemia cell cDNA expression libraries with anti-P.Tyr antibodies, the most frequently isolated classes of cDNA encode proteins related to the *cdc2*$^+$ and *nimA* cell cycle gene products. We have investigated the *cdc2*$^+$ related kinase in more detail, in an effort to understand its identification in a screen designed to identify tyrosine kinases.

The *cdc2*$^+$ family contains several yeast genes, including *cdc2* and its immediate *Saccharomyces cerevisiae* homologue *CDC28;KSS1*, which promotes passage of *S. cerevisiae* from G_1 to S phase of the cell cycle (Courchesne *et al.*, 1989); *FUS3*, which is required for pheromone-induced G_1 arrest and for conjugation (Elion *et al.*, 1990); and *YAK1*, which regulates cell growth, and is located downstream of cAMP-dependent protein kinase on the Ras/cAMP pathway (Garrett and Broach, 1989). In *Drosophila*, the segment-polarity gene *zw3* encodes two distinct and differentially expressed proteins which differ in their amino-terminal regions, but share a common C-terminal *cdc2*-like protein kinase domain (Siegfried *et al.*, 1990). In mammalian cells, in addition to *cdc2* itself, the mitogen activated protein (MAP) kinase (*ERK1*) has recently been shown to contain a *cdc2*-like kinase domain most similar to *KSS1* and *FUS3* (Boulton *et al.*, 1990). MAP kinase is implicated in signal transduction, since it is activated by a variety of mitogenic stimuli (Rossomondo *et al.*, 1988) and is capable of re-activating dephosphorylated S6 kinase-II (Sturgill *et al.*, 1988). MAP kinase itself is inactive unless phosphorylated on both tyrosine and threonine (Anderson *et al.*, 1990). In the sense that MAP kinase requires phosphorylation on both tyrosine and threonine for regulation of its kinase activity, it is similar to p34^{cdc2} of higher eukaryotes. Within its kinase domain, the *clk* gene product (Clk) is most closely related to *FUS3*. There is a sequence of 26 amino acids which is highly conserved among *cdc2* family members and quite distinct from the analogous sequences of most other protein kinases. As yet, we have no data as to the function of mammalian *clk*. However, the observation that *clk* is related to the *cdc2*$^+$ gene family raises the possibility that it is involved in regulating signal transduction or the cell cycle.

Clk is associated with both protein-serine/threonine and protein-tyrosine kinase activity

The most startling feature of the *clk* and *nek* cDNAs is that their products have all the sequence attributes of protein-serine/threonine kinases, despite the fact that they were isolated using anti-P.Tyr antibodies. The repeated isolation of *clk* and *nek* indicates that they have some unusual property, since the Friend cell libraries presumably contain a wealth of *bona fide* protein-serine/threonine kinase cDNAs. The Clk protein becomes phosphorylated on both tyrosine and serine/threonine *in vitro*. Since the phosphate in

these reactions is derived from ATP labeled in the γ position, the isolation of phosphotyrosine through some spurious mechanism other than direct protein phosphorylation is unlikely. A critical issue is whether the phosphorylation of TrpE−Clk on serine, threonine and tyrosine represents an intrinsic capacity of the Clk kinase domain to phosphorylate all three hydroxyamino acids, at least in an autophosphorylation reaction. Strong evidence in support of this notion is provided by the blot denaturation/renaturation kinase assay. In this experiment, the 85 kd TrpE−Clk protein became phosphorylated on tyrosine and serine/threonine after separation on a denaturing SDS−polyacrylamide gel and renaturation on a filter. It is difficult to explain this observation in any way other than to suggest that the TrpE−Clk protein has an intrinsic capacity to autophosphorylate on tyrosine as well as serine/threonine.

The *in vitro* tyrosine and serine/threonine phosphorylation of the 45 kd TrpE−Clk fragment, which itself apparently lacks an intact catalytic domain, suggests that Clk might be able to transfer phosphate to tyrosine and serine/threonine residues in *trans*. It is of interest that almost one third (47/148) of the residues in the unique N-terminal region of Clk are hydroxyamino acids. There is also a concentration of basic residues in this region (32/148). TrpE−Clk was also able to phosphorylate a random poly(glu,tyr) co-polymer, added as an exogenous substrate to an *in vitro* kinase reaction, confirming that it has protein-tyrosine kinase activity.

Protein kinase specificity

The Clk protein kinase domain contains the sequence elements considered diagnostic of protein serine/threonine kinases, in agreement with the finding that Clk does indeed phosphorylate serine and threonine. However, Clk also appears to possess intrinsic protein-tyrosine kinase activity. There are no obvious clues in the protein sequence to explain this observation. Within the motif HTDLKPEN, Clk has a threonine where most other kinases have arginine. Interestingly, the predicted S1 cDNA product has a serine at this site (L.Tannock, unpublished results). In addition, Clk has an unusual 19 amino acid insert, relative to other protein kinases, in between the HTDLKPEN sequence and the highly conserved DFG motif (Hanks *et al.*, 1988; Moran *et al.*, 1988). The observation that all protein kinases tested to date show a mutually exclusive preference for either serine/threonine or tyrosine, as well as the identification of sequence motifs diagnostic of each subclass, has suggested an intrinsic difference between the tyrosine- and serine/threonine-specific protein kinases. However, the evidence presented above suggests that Clk may be able to phosphorylate all three hydroxyamino acids. Other protein kinases with the sequence motifs of protein-serine/threonine kinases may possess similar properties; a protein kinase has recently been identified in *S.cerevisiae* which is phosphorylated on tyrosine *in vivo*, and is associated with both protein-serine and protein-tyrosine kinase activity *in vitro* (Dailey *et al.*, 1990). The *nek* product also has the appearance of a protein-serine/threonine kinase, and has no obvious unique sequence element in common with Clk. Experiments are in progress to determine whether the *nek* protein can phosphorylate tyrosine.

Two *Dictyostelium discoideum* protein kinases (DPYK 1 and 2) have recently been described whose catalytic domains phosphorylate only tyrosine when expressed in bacteria (Tan and Spudich, 1990). In screening a 15 h developmental *D.discoideum* expression library with anti-P.Tyr antibodies we have also isolated a cDNA identical to DPYK2 (K.Letwin, M.Aberman, G.Weeks and T.Pawson, unpublished results). The sequences of DPYK 1 and 2 can be considered as structural mosaics of protein-serine/threonine and protein-tyrosine kinase motifs, and yet specifically phosphorylate tyrosine *in vitro*. Taken with the properties of *clk*, these results suggest that we have yet to understand fully the rules governing the substrate specificities of protein kinases.

Possible functions of protein-serine/threonine/tyrosine kinases

The apparent ability of Clk to phosphorylate tyrosine may not be maintained *in vivo* in mammalian cells, or may be a tolerated aberration of no functional consequence. Assuming, however, that such multi-functional kinases are of biological significance, it is of interest to consider what role they might play. Both *clk* and *nek* are most closely related to a network of protein kinases that regulate the cell cycle. Some of these protein kinases may have developed a mechanism by which they are distinguished from the classical protein-tyrosine and protein-serine/threonine kinases, $p34^{cdc2}$ of higher eukaryotes must be phosphorylated on both tyrosine and threonine to be held in an inactive state, and thereby allow correct timing of mitosis; conversely, the *cdc2*-like MAP kinase must be phosphorylated on both tyrosine and threonine to be activated. One interpretation of these data is that at least two individual kinases of different specificities are required either for the inhibition of $p34^{cdc2}$ or the activation of MAP kinase. An alternative possibility is that these protein kinases are themselves controlled by protein kinases with the ability to phosphorylate serine/threonine as well as tyrosine. Such protein-serine/threonine/tyrosine kinases would therefore represent a new class of protein kinase, and would presumably have very specific substrates. We are presently analyzing the *clk* and *nek* proteins expressed in mammalian cells to determine whether they possess such attributes.

Materials and methods

Antibodies

To isolate anti-phosphotyrosine antibodies, rabbit polyclonal antiserum against a polymer of phosphotyrosine, alanine, glycine and keyhole limpet hemocyanin was raised and affinity purified as originally described by Kamps and Sefton (1988). Rabbit anti-TrpE antiserum was raised to the 37 kd product of the parental pATH expression plasmid.

Construction of cDNA libraries and isolation of cDNA clones

Two λgt11 cDNA expression libraries were constructed from 5 μg poly(A)$^+$ RNA isolated from the erythroleukemia cell lines CB7 (Shibuya and Mak, 1982) and DP28-9 (Ben-David *et al.*, 1988), using a Pharmacia cDNA synthesis kit. Screening of λgt11 expression libraries using anti-phosphotyrosine antibody was carried out as described previously (Letwin *et al.*, 1988).

Subcloning and nucleotide sequence determination

DNA was prepared from plaque purified phage according to standard procedures using lambdasorb phage adsorbent and subcloned into pGEM-5Zf(+) or pGEM-7Zf(+). Clones were grouped together based on partial DNA sequence analysis and cross-hybridization. For complete sequence determination, nested deletions spanning the cDNA insert of the desired clone were generated using the method of Henikoff (1987). These constructs were rescued as ssDNA following superinfection of bacteria with

the coliphage M13K07 and sequenced by the dideoxynucleotide chain termination method using sequenase enzyme, reagents and protocols supplied by United States Biochemicals Corporation. Sequences were obtained for both strands.

Construction of the trpE−clk bacterial expression vector

The plasmid pE3-2 was constructed by subcloning the 1.7 kb *Eco*RI cDNA insert of λE3 into the *Eco*RI site of pGEM-7Zf(−). The expression vector ptE3 was constructed by subcloning the 1.4 kb *Hin*cII−*Hin*dIII fragment of pE3-2 into the *Sal*I (blunted with Klenow)−*Hin*dIII sites of pATH-2. This construct produced an 85 kd TrpE−Clk fusion protein composed of the first 330 amino acids of the TrpE protein and the C-terminal 414 amino acids of the Clk protein.

Immunoblotting bacterial lysates

Escherichia coli DH5α harboring the expression plasmid ptE3 or a control plasmid pATH-2 were grown and induced as described previously (Moran *et al.*, 1988). Following a 3 h induction period, a 1 ml culture was pelleted, and the cells were lysed in 50 μl cracking buffer [10 mM sodium phosphate, pH 7.2; 1% (v/v) β-mercaptoethanol, 1% (w/v) SDS, 6 M urea] and vortexed at room temperature for 30 min. The lysate was diluted with an equal volume of 2 × SDS sample buffer and 5 μl aliquots were resolved on 7.5% SDS−polyacrylamide gels and transferred to nitrocellulose.

Blotted proteins were blocked in TBST [10 mM Tris−HCl, pH 8.0; 150 mM NaCl; 0.05% (v/v) Tween-20] containing 5% (w/v) bovine serum albumin overnight. Blots were then incubated with anti-P.Tyr or anti-TrpE antibodies in TBST for 1 h, washed with TBST, incubated with a goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma), washed with TBST and immersed in alkaline phosphatase buffer containing the chromogenic substrates NBT and BCIP.

In vitro kinase reaction

Following a 3 h induction period, cells from 5 ml cultures of *E.coli* DH5α harboring the expression vector ptE3 or the control plasmid pATH-2 were pelleted, resuspended in 1 ml TBS and lysed by sonication. Lysates were clarified by centrifugation at 13 000 *g* for 30 min. Proteins were isolated by immunoprecipitation with either a polyclonal anti-TrpE antiserum or affinity-purified anti-P.Tyr antibodies, and heat-inactivated *Staphylococcus aureus*. Immunoprecipitates were washed once with KLB and twice with kinase reaction buffer (50 mM HEPES, pH 7.0; 10 mM $MnCl_2$; 10 mM $MgCl_2$), resuspended in 25 μl kinase reaction buffer containing 5 μCi [γ-^{32}P]ATP (20 Ci/mmol) and incubated at room temperature for 15 min. Reactions were terminated by addition of an equal volume of 2 × SDS sample buffer and the immunoprecipitates were released by incubation at 30°C for 15 min. The kinase reaction supernatant was then resolved by SDS−PAGE, transferred to PVDF membranes (Immobiolon; Millipore Corporation) and ^{32}P-labeled proteins visualized by autoradiography. *In vitro* kinase reactions using an exogenous substrate were conducted essentially as described above except that poly(glu, tyr) 4:1 (Sigma) was included in the reaction buffer at a concentration of 100 μg/ml (Braun *et al.*, 1984).

Phosphoamino acid analysis

Pertinent regions of the PVDF membrane were excised and the ^{32}P-labeled protein hydrolyzed in 6 M HCl for 60 min at 110°C (Kamps and Sefton, 1989). Supernatants were lyophilized, mixed with non-radioactive phosphoamino acid standards, and analyzed by 2-dimensional electrophoresis (pH 1.9 followed by pH 3.5) on thin-layer cellulose plates.

Kinase renaturation assay

The denaturation−renaturation protocol was carried out essentially as described by Ferrell and Martin (1989, 1990). Whole cell lysates from 50 to 500 μl induced bacteria were resolved by SDS−PAGE and transferred to PVDF membranes. Blotted proteins were immersed in denaturation solution (6 M guanidinium chloride, 50 mM Tris−HCl, pH 8.3, 50 mM DTT, 2 mM EDTA) for 60 min at 4°C, washed briefly with renaturation buffer (20 mM Tris−HCl, pH 7.5; 150 mM NaCl; 2 mM DTT; 2 mM EDTA; 0.1% Nonidet P-40, 2% glycerol) and immersed overnight at 4°C in renaturation buffer. Blots were blocked by immersing in renaturation buffer containing 0.2% polyvinylpyrrolidone, 0.2% Ficoll and 20 mM sodium pyrophosphate for 60 min at room temperature, overlaid with reaction buffer (30 mM Tris−HCl, pH 7.5; 10 mM $MgCl_2$; 10 mM $MnCl_2$; [γ-^{32}P]ATP [Amersham; 3000 Ci/mmol, 50 μCi/ml, 1 ml per 10 cm^2 blot area]) for 30 min, and then washed extensively with TBS prior to autoradiography.

RNA extraction and Northern blotting

Total cellular RNA from different mouse tissues was isolated by the lithium chloride precipitation procedure (Auffray and Rougeon, 1980). The isolated RNA was extracted three times with water saturated phenol/chloroform (1:1) and once with chloroform. The final aqueous phase was precipitated with ethanol and stored in 100% ethanol at −70°C. Total RNA (20 μg) was fractionated using formaldehyde denaturing gel electrophoresis and then blotted onto nitrocellulose paper. The filter was hybridized with a random primed *clk* cDNA probe corresponding to the full length E3 clone (Feinberg and Vogelstein, 1983).

Acknowledgements

We thank R.Lindberg and T.Hunter for supplying phosphotyramine, C.A.Koch for anti-TrpE antisera, B.A.Taylor for help with the chromosomal mapping and A.Ochi for lymphoid cell lines. We are grateful to G.Cheong for technical assistance, J.Copeland for help in the analysis of cDNA clones and D.Queensborough for preparation of the manuscript. K.L. holds a fellowship from the National Cancer Institute of Canada (NCIC). Y.B.-D. is supported by the Leukemia Research Fund. T.P. is a Terry Fox Cancer Research Scientist of the NCIC. A.B. is a McClaughlin Scientist. This work was supported by a grant from Bristol-Myers-Squibb.

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Received on October 1, 1990; revised on November 28, 1990

Note added in proof

The **clk** sequence data will appear in the EMBL/Genbank/DDBJ nucleotide sequence databases under the accession number X57186.