Pro-Leu-Ser/Thr-Pro Is a Consensus Primary Sequence for Substrate Protein Phosphorylation

CHARACTERIZATION OF THE PHOSPHORYLATION OF c-myc AND c-jun PROTEINS BY AN EPIDERMAL GROWTH FACTOR RECEPTOR THREONINE 669 PROTEIN KINASE*

(Received for publication, February 6, 1991)

Elvira Alvarez, Ingrid C. Northwood, Fernando A. Gonzalez, Debra A. Latour, Cory Abate, Tom Curran, and Roger J. Davis

From the *Howard Hughes Medical Institute, †Program in Molecular Medicine, ‡Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01605 and the §Department of Molecular Oncology and Virology, Roche Institute of Molecular Biology, Nutley, New Jersey 07110

A growth factor-stimulated (MAP2-related) protein kinase, ERT, that phosphorylates the epidermal growth factor receptor at Thr669 has been purified from KB human tumor cells by Northwood and co-workers (Northwood, I. C., Gonzalez, F. A., Wartmann, M., Raden, D. L., and Davis, R. J. (1991) J. Biol. Chem. 266, 15266-15276). The ERT protein kinase has a restricted substrate specificity, and the structural determinants employed for substrate recognition by this enzyme have not been defined. As an approach toward understanding the specificity of substrate phosphorylation, we have used an in vitro assay to identify additional substrates for the ERT protein kinase. In this report we describe two novel substrates: (a) the human c-myc protein at Ser62 and (b) the rat c-jun protein at Ser62. Alignment of the primary sequences surrounding the phosphorylation sites located within the epidermal growth factor receptor (Thr669), Myc (Ser62), and Jun (Ser62) demonstrated a marked similarity. The observed consensus sequence was Pro-Leu-Ser/Thr-Pro. We propose that this sequence forms part of a substrate structure that is recognized by the ERT protein kinase.

Treatment of cultured cells with epidermal growth factor (EGF), platelet-derived growth factor, phorbol ester, or serum causes a marked increase in the state of phosphorylation of the EGF receptor at Thr669 (1-3). The increase in phosphorylation is caused by the stimulation of a protein kinase activity that can be detected in cell extracts (2). This protein kinase activity is accounted for by two distinct enzymes: 1) the MAP2 protein kinase and 2) a novel serine/threonine protein kinase that we designate ERT (EGF Receptor Thr669) protein kinase (4).

Recently, the ERT protein kinase was purified from KB human tumor cells (4). In vitro phosphorylation assays demonstrated that the purified ERT protein kinase has a very restricted substrate specificity (4). Indeed, the EGF receptor is the only substrate for the ERT protein kinase that has been characterized in detail (2-5). Phosphorylation of the EGF receptor at Thr669 regulates both tyrosine phosphorylation and receptor endocytosis (5). This phosphorylation of the EGF receptor may therefore be physiologically significant, but it is likely that there are additional substrates for the ERT protein kinase. This is because growth factor-stimulated ERT protein kinase activity can be detected in cells that do not express EGF receptors (2, 6). The marked extent and rapid kinetics of activation of the ERT protein kinase by growth factors suggest that this enzyme may have an important function during signal transduction (2, 6). Regulatory proteins within signal transduction pathways therefore represent potential substrates for the ERT protein kinase.

The purpose of the experiments reported here was to examine the recognition of substrate proteins by the ERT protein kinase. The experimental strategy that we employed was to test potential substrate proteins in an in vitro phosphorylation assay using the purified ERT protein kinase. We report here the identification of two protein substrates: the products of the proto-oncogenes c-myc and c-jun. Alignment of the primary sequences surrounding the phosphorylation sites located within the EGF receptor, Jun, and Myc indicates a consensus sequence Pro-Leu-Ser/Thr-Pro. We propose that this sequence forms part of a substrate structure that is recognized by the ERT protein kinase.

EXPERIMENTAL PROCEDURES

Materials—[35S]Methionine and [32P]dATP were obtained from Amersham Corp. [32P]ATP was from Du Pont-New England Nuclear. [γ-32P]ATP was prepared using a Gamma-Prep A kit (Promega Biotech) according to the manufacturer's directions. The ERT protein kinase was purified from KB cells as described previously (4).

Nuclear extracts were prepared from KB cells as described (7). Tag polymerase was from Perkin-Elmer Cetus. Synthetic peptides were obtained from the Peptide Synthesis Core Facility (University of Massachusetts Medical School, Worcester, Massachusetts 01605).

* These studies were supported in part by Grants CA39240 and GM37845 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of a postdoctoral fellowship from the National Science Foundation.

** To whom correspondence should be addressed: Howard Hughes Medical Inst., Program in Molecular Medicine, University of Massachusetts Medical School, 375 Plantation St., Worcester, MA 01605.

1 The abbreviations used are: EGF, epidermal growth factor; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; ERT protein kinase, protein kinase that phosphorylates the EGF receptor at Thr669; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Jun, the protein product of the c-jun gene; MAP2 protein kinase, growth factor-stimulated protein kinase that phosphorylates microtubule-associated protein 2; Myc, the protein product of the c-myc gene.

2 The EGF receptor phosphorylation site Thr669 is conserved in c-erbB2 (38). It is therefore likely that the c-erbB2 gene product is also a substrate for the ERT protein kinase.
EPT Protein Kinase Substrate Specificity

Massachusetts Medical School. Restriction enzymes were from Boehringer Mannheim.

Plasmid Construction and Purification of Bacterially Expressed Proteins—Full-length Jun (Jun(1–334)) and a truncated Jun polypeptide containing amino acids 224–334 (Jun(224–334)) were expressed in Escherichia coli as hexahistidine fusion proteins and purified by nickel affinity chromatography as described (8, 9). Deletion of the coding sequence corresponding to amino acids 245–247 in Jun(224–334) was achieved using a polymerase chain reaction strategy (10). The sequence of the mutated gene was confirmed by dideoxynucleotide sequencing (11).

Glutathione S-transferase (GST) fusion proteins were constructed in the vector pGEX-3X (Pharmacia LKB Biotechnology Inc). The coding region of the human c-myc gene corresponding to exons 2 (amino acids 2–251) was isolated from a genomic clone (American Type Culture Collection 41010) using the polymerase chain reaction and the oligonucleotide primers, 5′ ggc ggg acc aac cag acc acc acc acc acc cag 3′ and 5′ ggc ggg acc aac cag acc acc acc acc acc cag 3′. The amplified DNA was cloned as a blunt-end fragment into the vector pGEX-3X. A point mutation was introduced at codon 62 using a polymerase chain reaction strategy (10), and the sequence was confirmed using the dideoxynucleotide method and synthetic oligonucleotide primers (11). The fusion protein was isolated from bacteria induced with 2 h with 0.4 mM isopropyl-I-thiogalactoside by chromatography using glutathione-agarose as described (12). These fusion proteins were designated GST/Myc and GST/[Ala12]Myc, GST/[Ala4]Myc, and GST/[Ala67]Myc.

CDX1 (1–147) fusion proteins were prepared using the vector pSG424 (13). A DNA fragment of the human c-myc gene corresponding to amino acids 2–103 was isolated using the polymerase chain reaction and the oligonucleotide primers, 5′ ggc ggg acc aac cag acc acc acc acc acc cag 3′ and 5′ ggc ggg acc aac cag acc acc acc acc acc cag 3′. The amplified DNA was cloned as a blunt-end fragment into pUC13 at the Smal site. Point mutations were introduced at codons 62, 64, and 67 using a polymerase chain reaction strategy (10). The sequence of the amplified DNA was confirmed using the dideoxynucleotide method and synthetic oligonucleotide primers (11). The c-myc fragments were then excised from pUC13 by restriction endonuclease digestion and cloned as BamHI-XbaI fragments into the polylinker of the vector pSG424. The fusion proteins were transiently expressed in COS-1 cells and were designated GALA/Myc, GALA/[Ala12]Myc, GALA/[Ala4]Myc, and GALA/[Ala67]Myc.

Transient Expression of Fusion Proteins in COS-1 Cells—COS-1 cells in 100-mm dishes were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum. Plasmid DNA (10 μg) was transfected using the DEAE-dextran method (14). After 30 h of incubation the culture medium was aspirated and replaced with 5 ml of labeling medium supplemented with 1% fetal calf serum: (a) modified Eagle’s medium containing 50 μM [35S]methionine (20 μCi/ml) or (b) phosphate-free mod Eagle’s medium containing 0.4 mM [32P]phosphate. After 18 h of additional incubation, the cells were lysed in 25 mM HEPES (pH 7.5), 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 5 mM EGTA, 50 mM NaF, 500 mM NaCl, 100 μM Na3VO4, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The lysates were clarified by centrifugation at 100,000 × g, and the supernatant was incubated for 60 min at 22 °C with 5 μl of rabbit anti-GALA antiserum prebound to 20 μl of protein A-Sepharose. The immunoprecipitates were washed three times with lysis buffer, washed once with 25 mM HEPES (pH 7.5), 0.1 mM EGTA, and analyzed by polyacrylamide gel electrophoresis.

Phosphorylation of Synthetic Peptides Based on the Primary Sequence of Jun and Myc by the Purified EPT Protein Kinase—Synthetic peptides were phosphorylated at 22 °C in a buffer containing 25 mM HEPES (pH 7.4), 10 mM MgCl2, 1 mg/ml synthetic peptide, purified EPT protein kinase (8 μg), and γ32P-ATP (40 μCi/nmol) in a final volume of 25 μl. The phosphorylation reaction was terminated after 20 min by the addition of 5 μl of 90% formic acid. The phosphorylated synthetic peptide was isolated by electrophoresis (4 °C) for 3 h at 500 V on a 100-μm cellulose thin layer plate using 30% (v/v) formic acid as solvent. The phosphorylated peptide was isolated from the plate by extraction with 30% formic acid and lyophilized.

Phosphorylation of Jun and Myc Proteins by the EPT Protein Kinase—Jun and Myc proteins (200 ng) were phosphorylated at 22 °C in a buffer containing 25 mM HEPES (pH 7.4), 10 mM MgCl2, purified EPT protein kinase (8 μg), and γ32P-ATP (40 μCi/nmol) in a final volume of 25 μl. The phosphorylation reaction was terminated after 20 min by the addition of 100 μl of Laemmli sample buffer. Phosphorylated proteins were analyzed by polyacrylamide gel electrophoresis (15) and autoradiography.

Phosphoamino Acid Analysis—Phosphoamino acid analysis was performed by partial acid hydrolysis (1 h at 110 °C in 6 M HCl) and thin layer electrophoresis as described (16, 17).

Tryptic [32P]Phosphopeptide Mapping—Phosphorylated proteins were eluted from gel slices using sodium dodecyl sulfate, precipitated with trichloroacetic acid, and oxidized with performic acid as described (18). The sample was digested with 1 μg of tosylphenylalanyl chloromethyl ketone-treated trypsin in 100 mM N-ethylmorpholine (pH 8.0). After 5 h, a second addition of trypsin was made, and the incubation was allowed to proceed for an additional 19 h. (In some experiments 100 ng of trypsin was used to confirm the identity of limit tryptic phosphopeptides.) Phosphopeptide mapping was performed by two-dimensional separation on 100-μm cellulose thin layer plates as described (19). The first dimension was electrophoresis in 30% formic acid at 500 V for 2 h at 4 °C. The second dimension was chromatography in butan-1-ol/pyridine/acetic acid/water (15:10:12). Electrophoresis was performed in the horizontal dimension (cathode at right side) and the chromatography was performed in the vertical dimension. The origin is located in the lower left corner of each panel (see figures).

Measurement of EPT Protein Kinase Activity—KB cells were obtained from the American Type Culture Collection and were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum. The KB cells were seeded in 100-mm dishes and were grown to confluence. The cells were washed with serum-free medium and were incubated for 30 min at 37 °C. After treatment for 5 min with 10 nM EGF, the cells were harvested. Cytosolic extracts were prepared by the method of Northwood and Davis (6). Nuclear extracts were prepared using the method of Lee et al. (7). Protein was determined using the Bradford dye binding assay (Bio-Rad) using bovine serum albumin as standard. Protein kinase assays were performed using a procedure that we have previously described (4). The synthetic peptide substrate used was Lys-Arg-Glu-Leu-Val-Glu-Pro-Leu-ThrAsp-Pro-Ser-Glu-Ala-Pro-Gln-Ala-Leu-Arg. The assay was performed using 1 μg of HEPES (pH 7.4), 10 mM MgCl2, 50 μM γ32P-ATP (10 μCi/nmol), 1 mg/ml synthetic peptide in a final volume of 25 μl. The reactions were terminated after 20 min at 22 °C by the addition of 10 μl of 90% formic acid containing 50 mM ATP. Control experiments demonstrated that the phosphorylation reaction was linear with time for 30 min. The phosphorylated synthetic peptide was isolated by electrophoresis and characterization of the phosphorylation of these proteins.

ERT Protein Kinase Substrate Specificity

A mitogen-stimulated protein kinase (EPT) that phosphorylates the EGF receptor at Thr441 has been purified from KB human tumor cells (4). To identify substrates for this protein kinase we used an in vitro assay to examine the phosphorylation of purified proteins by the isolated protein kinase. During the initial screening of potential substrates two proteins were found to be markedly phosphorylated: Myc and Jun. Further experiments were designed to provide a detailed characterization of the phosphorylation of these proteins.

To identify substrates for this protein kinase we used an in vitro assay to examine the phosphorylation of purified proteins by the isolated protein kinase. No protein phosphorylation was observed when the EPT protein kinase was incubated with γ32P-ATP. The absence of phosphorylation is consistent with previous observations that the EPT protein kinase does not autophosphorylate and that the purified enzyme is not significantly contaminated with exogenous substrates (4). Addition of Jun to the incubation resulted in marked phosphorylation of the Jun protein (Fig. 1A, lane C). Phosphoamino acid analysis indicated that Jun was phosphorylated on serine residues (Fig.
Jun proteins were analyzed by polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate. An autoradiograph of the protein kinase. The phosphorylation of wild-type Jun(1-334) phosphorylated by the ERT protein kinase. It was observed that the deletion of residues 245–247 blocked the phosphorylation of Jun (Fig. 1A, lane B). This observation is consistent with the hypothesis that Ser246 is the major site of Jun phosphorylation. (b) A synthetic peptide based on the primary sequence of Jun was prepared: EEPQTVMPGETPPLS246PIDMESQER. This peptide corresponds to the predicted Jun tryptic peptide that contains Ser246. It was observed that the peptide was a substrate for phosphorylation by the purified ERT protein kinase (data not shown). Phosphoamino acid analysis demonstrated the presence of [32P]phosphoserine (data not shown). Analysis of the phosphorylated synthetic peptide by two-dimensional peptide mapping demonstrated the presence of three [32P]phosphopeptides (Fig. 2). These phosphopeptides co-migrated during electrophoresis and therefore do not correspond to forms of the synthetic peptide phosphorylated at different numbers of sites. However, the phosphopeptides were resolved by chromatography indicating that the peptides differ in hydrophobicity. In several experiments it was found that the relative yield of the three phosphopeptides was variable (data not shown). This observation suggested that the peptide may be degraded during sample preparation. One possibility is that the extraction and lyophilization of the synthetic peptide modified the site(s) of phosphorylation.

Fig. 1. Jun is a substrate for phosphorylation by the ERT protein kinase. Panel A, purified ERT protein kinase (~5 ng) was incubated without (lane A) or with 200 ng of wild-type and mutated Jun proteins (lanes B–D) in 25 mM HEPES (pH 7.4), 10 mM MgCl2, and 50 μM [γ-32P]ATP (10 μCi/nmol) in a final volume of 25 μL. After 20 min of incubation at 22 °C, the reaction was terminated by the addition of 100 μL of Laemmli sample buffer. The phosphorylated proteins were analyzed by polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate. An autoradiograph of the dried gel is presented. The phosphorylation of wild-type Jun(1–334) is shown in lane C. Truncated Jun(224–334) was also phosphorylated by the ERT protein kinase (lane D). However, no phosphorylation of the truncated Jun(224–334) containing an internal deletion of residues 245–247 was detected (lane B). Panel B, phosphoamino acid analysis of wild-type Jun(1–334) phosphorylated by the ERT protein kinase was performed. Panel C, the phosphorylation state of wild-type and mutated forms of Jun was examined by tryptic phosphopeptide mapping. The electrophoretic dimension (cathode at right) and the chromatography dimension are shown. The origin is marked with a cross at the lower left corner of each peptide map.

1B). No threonine or tyrosine phosphorylation was detected (Fig. 1B).

In order to localize the site(s) of phosphorylation of Jun by the ERT protein kinase, we investigated the phosphorylation of Jun proteins containing NH2-terminal deletions. It was observed that the deletion of residues 1–223 did not block Jun phosphorylation (Fig. 1A, lane D). Tryptic [32P]phosphopeptide maps of the full-length and truncated Jun proteins were identical (Fig. 1C). Thus, the Jun phosphorylation site(s) are located in the COOH terminus of the protein between residues 224 and 334.

Visual inspection of the primary sequence of the COOH terminus of Jun indicated a region that was homologous to the sequence of the EGF receptor surrounding the phosphorylation site Thr269. The predicted Jun phosphorylation site was Ser246. The following two experiments were performed to test the hypothesis that Jun was phosphorylated at Ser246.

(a) If Ser246 is a site of Jun phosphorylation, deletion of this residue would be expected to markedly reduce the phosphorylation of Jun by the ERT protein kinase. It was observed that the deletion of residues 245–247 blocked the phosphorylation of Jun (Fig. 1A, lane B). This observation is consistent with the hypothesis that Ser246 is the major site of Jun phosphorylation.

(b) A synthetic peptide based on the primary sequence of Jun was prepared: EEPQTVMPGETPPLS246PIDMESQER. This peptide corresponds to the predicted Jun tryptic peptide that contains Ser246. It was observed that the peptide was a substrate for phosphorylation by the purified ERT protein kinase (data not shown). Phosphoamino acid analysis demonstrated the presence of [32P]phosphoserine (data not shown). Analysis of the phosphorylated synthetic peptide by two-dimensional peptide mapping demonstrated the presence of three [32P]phosphopeptides (Fig. 2). These phosphopeptides co-migrated during electrophoresis and therefore do not correspond to forms of the synthetic peptide phosphorylated at different numbers of sites. However, the phosphopeptides were resolved by chromatography indicating that the peptides differ in hydrophobicity. In several experiments it was found that the relative yield of the three phosphopeptides was variable (data not shown). This observation suggested that the peptide may be degraded during sample preparation. One possibility is that the extraction and lyophilization of the synthetic peptide modified the site(s) of phosphorylation.
peptide in formic acid causes partial deamidation of the two glutamine residues present in the synthetic peptide. However, no direct evidence for this hypothesis was obtained.

Comparison of the peptide maps of the phosphorylated synthetic peptide (Fig. 2) with maps of Jun (Fig. 1) indicated a marked similarity. In each case three major phosphopeptides were observed that co-migrated during electrophoresis and were resolved by chromatography. This similarity suggested that the phosphopeptides present in these maps may be identical. To test this hypothesis we performed mixing experiments to examine whether phosphopeptides from the maps co-migrated during two-dimensional separation. In initial experiments it was found that the peptides did not co-migrate (Fig. 2C). However, on close inspection it was observed that the phosphorylated synthetic peptide did co-migrate with a set of three minor peptides present in the maps of Jun (Fig. 2). We therefore considered the possibility that the major phosphopeptides present in the maps of the Jun protein may represent the products of a partial tryptic digestion. The electrophoretic mobility of the peptides was consistent with this hypothesis. In order to characterize the limit tryptic [32P] phosphopeptides we repeated the proteolytic digestion in the presence of a higher concentration of trypsin. It was observed that three limit tryptic phosphopeptides were obtained (Fig. 2D). These limit tryptic [32P] phosphopeptides co-migrated with the [32P] phosphopeptide synthetic peptide (Fig. 2F). The observed co-migration suggests an identity between the synthetic peptide and the tryptic peptide containing the Jun phosphorilation site.

We conclude from the results of the deletion studies (Fig. 1) and comparative [32P] phosphopeptide mapping (Fig. 2) that Jun is phosphorylated at Ser\(^{246}\) by the ERT protein kinase.

**Myc Is Phosphorylated at Ser\(^{246}\) by the ERT Protein Kinase**—The phosphorylation of Myc by the purified ERT protein kinase was examined in an in vitro assay using a bacterially expressed GST/Myc fusion protein as a substrate.

No phosphorylation of glutathione S-transferase by the ERT protein kinase was observed (data not shown). However, the GST/Myc fusion protein was marked phosphorylated (Fig. 3A). Phosphoamino acid analysis demonstrated the presence of phosphoserine, but no phosphothreonine or phosphotyrosine was detected (Fig. 3B). To further characterize the phosphorylation we performed phosphopeptide mapping after tryptic digestion of the phosphorylated Myc protein. Fig. 4 shows that two tryptic [32P] phosphopeptides were resolved by two-dimensional separation on cellulose thin layer plates. Together, these data indicate that Myc contains at least one serine residue that is phosphorylated by the ERT protein kinase.

---

The presence of partially digested peptides in tryptic phosphopeptide maps is not uncommon and has been reported previously. Two examples of proteolytic sites that are cleaved very slowly by trypsin are provided by studies of the phosphorylation of the EGF receptor (17, 39) and the transferrin receptor (19) by protein kinase C.

The site of phosphorylation of rat Jun reported here is Ser\(^{246}\). This site is equivalent to Ser\(^{245}\) in human Jun (33, 40). The primary sequence surrounding this phosphorylation site is identical between the rat and human sequences.

In initial experiments a glutathione S-transferase fusion protein was prepared using the full-length Myc protein. However, this protein was found to be insoluble and was not used for in vitro protein kinase assays. Glutathione S-transferase fusion proteins were therefore prepared containing the NH\(_2\) or the COOH domains of Myc. The COOH domain fusion protein was not phosphorylated by the purified ERT protein kinase (E. Alvarez, unpublished observation). In contrast, the NH\(_2\) domain fusion protein (GST/Myc) was found to be a substrate (Fig. 3A).

---

To identify the site(s) of phosphorylation of Myc, we compared the primary sequence of Myc with the sequence of the EGF receptor surrounding Thr\(^{294}\). This analysis suggested that Ser\(^{246}\) was a potential site of myc phosphorylation. The hypothesis that Myc was phosphorylated at Ser\(^{246}\) was tested using the following two experimental approaches. (a) Ser\(^{246}\) was substituted with an Ala residue, and the effect of this mutation on the phosphorylation of the GST/Myc fusion protein was investigated. Fig. 3A shows that the GST/[Ala\(^{246}\)]

---

**Fig. 3.** Myc is a substrate for phosphorylation by the ERT protein kinase. Panel A, purified ERT protein kinase (~5 ng) was incubated without (lane 1) or with GST/[Ala\(^{246}\)]Myc (200 ng, lane 2) or with GST/Myc (200 ng, lane 3) in 25 \(\mu\)l of 25 mM HEPES (pH 7.4), 10 mM MgCl\(_2\), and 50 \(\mu\)M [\(\gamma\)-\(^32\)P]ATP (10 \(\mu\)Ci/nmol). After 20 min of incubation at 22 °C, the reaction was terminated by the addition of 100 \(\mu\)l of Laemmli sample buffer. The phosphorylated proteins were analyzed by polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate. An autoradiograph of the dried gel is presented. Panel B, the phosphorylated GST/Myc protein was examined by phosphoamino acid analysis.

---

**Fig. 4.** Analysis of Myc phosphorylation by phosphopeptide mapping. The synthetic peptide KKFELLPTPPLS\(^{246}\)PSRR and the bacterially expressed fusion protein GST/Myc were phosphorylated by the purified ERT protein kinase using \([\gamma\)-\(^32\)P]ATP. The synthetic peptide and GST/Myc were isolated by cellulose thin layer and polyacrylamide gel electrophoresis, respectively. After digestion with trypsin, the \([\gamma\)-\(^32\)P] phosphopeptides obtained were analyzed by peptide mapping. Panel A presents an autoradiograph of a phosphopeptide map of the synthetic peptide (382 cpm). The results obtained for GST/Myc (366 cpm) are shown in panel B. A mixture of the synthetic peptide (184 cpm) and GST/Myc (172 cpm) was also analyzed (panel C). The exposure time used for autoradiography was 38 h at ~80 °C. The electrophoretic dimension (cathode at right) and the chromatography dimension are shown. The origin is marked with a cross at the lower left corner of each peptide map.
Myc fusion protein was not a substrate for phosphorylation by the ERT protein kinase. The lack of phosphorylation of the mutated protein suggests that the site of Myc phosphorylation was Ser\(^{62}\). (b) A synthetic peptide corresponding to the primary sequence surrounding Myc Ser\(^{62}\) was prepared: KKFELLPTPPS\(^{65}\)PSRR. This peptide was found to be a substrate for phosphorylation by the ERT protein kinase (data not shown). Phosphoamino acid analysis demonstrated the presence of phosphoserine, but not phosphothreonine or phosphotyrosine (data not shown). A single \([^{32}\text{P}]\)phosphopeptide with a high electrophoretic mobility was detected by twodimensional phosphopeptide mapping (data not shown). The synthetic peptide contains 4 lysine and arginine residues. We therefore digested the phosphorylated synthetic peptide with trypsin and examined the results of the proteolytic digestion by phosphopeptide mapping. Two tryptic phosphopeptides were obtained that were resolved by electrophoresis (Fig. 4).

The two peptides were probably the result of partial proteolysis because of the poor exopeptidase activity of trypsin.\(^6\) Mixing experiments demonstrated that the tryptic \([^{32}\text{P}]\)phosphopeptides derived from the synthetic peptide co-migrated with the \([^{32}\text{P}]\)phosphopeptides obtained by trypsin digestion of the GST/Myc fusion protein (Fig. 4). The observed co-migration suggests an identity between the tryptic phosphopeptides obtained from the synthetic peptide and the Myc protein.

The results of point mutation (Fig. 3) and comparative phosphopeptide mapping (Fig. 4) demonstrate that Ser\(^{62}\) is a site of Myc phosphorylation by the ERT protein kinase.

**Phosphorylation of Myc Ser\(^{62}\) in Intact Cells—The in vitro phosphorylation of Jun (Ser\(^{62}\)) and Myc (Ser\(^{62}\)) by the ERT protein kinase suggests that these proteins may be phosphorylated in intact cells. However, the demonstration of an in vitro phosphorylation does not necessarily imply that this phosphorylation will occur in vivo. It was therefore important to establish whether this phosphorylation could be found in intact cells. Recently, Ser\(^{62}\) has been demonstrated to be an in vivo site of Jun phosphorylation (20), but little information about the in vivo phosphorylation of Myc is available (21). We therefore investigated whether Myc Ser\(^{62}\) was phosphorylated in intact cells.

The experimental approach that we employed was to construct a fusion protein between a fragment of the yeast transcription factor GAL4 (residues 1–147) and Myc. It has previously been documented that this fusion protein is correctly localized in the nucleus and is functional as a transcriptional activator (22). After expression in COS cells,\(^6\) the GAL4/Myc fusion protein was markedly phosphorylated (Fig. 5). In contrast, GAL4(1–147) was not detectably phosphorylated (Fig. 6B). Thus, the observed phosphorylation is specific to the Myc fusion protein. To investigate whether the GAL4/Myc fusion protein was a substrate for an EGF-stimulated protein kinase we investigated the effect of EGF on the phosphorylation state of the GAL4/Myc fusion protein. Fig. 5 shows that EGF treatment caused a rapid increase in GAL4/Myc phosphorylation. Phosphoamino acid analysis demonstrated the presence of \([^{32}\text{P}]\)phosphoserine and some \([^{32}\text{P}]\)phosphotyrosine (Fig. 6C). The Myc protein was therefore phosphorylated at more than one site.

Phosphopeptide mapping of the Myc fusion protein isolated from EGF-treated cells indicated the presence of two major tryptic \([^{32}\text{P}]\)phosphopeptides and several minor \([^{32}\text{P}]\)phosphopeptides (Fig. 6D). The two major \([^{32}\text{P}]\)phosphopeptides co-migrated with the two \([^{32}\text{P}]\)phosphopeptides derived by tryptic digestion of the synthetic peptide KKFELLPTPPS\(^{65}\)PSRR phosphorylated in vitro by the ERT protein kinase (data not shown). The phosphorylation site located in the synthetic peptide corresponds to Ser\(^{62}\) (Figs. 3 and 4). These data indicate that Ser\(^{62}\) is the major site of phosphorylation of the Myc fusion protein in COS-1 cells.

To confirm that Myc Ser\(^{62}\) was phosphorylated in intact cells we investigated the effect of point mutations on the phosphorylation state of the Myc fusion protein. It was observed that the substitution of Ser\(^{62\,}\) with Ala caused no significant change in the level of phosphorylation (Fig. 6B) or the \([^{32}\text{P}]\)phosphopeptide map obtained after tryptic digestion (Fig. 6D). Similar results were obtained after the replacement of Ser\(^{62}\) with Ala (data not shown). However, replacement of Ser\(^{62}\) with Ala caused a decrease in the level of phosphorylation of the Myc fusion protein. Tryptic peptide mapping demonstrated that the two major \([^{32}\text{P}]\)phosphopeptides observed in maps of the wild-type protein were absent in maps of the mutant [Ala\(^{62}\)]Myc fusion protein (Fig. 6D). These data strongly support the hypothesis that Ser\(^{62}\) is phosphorylated in intact cells.

**Nuclear Localization of ERT Protein Kinase Activity—**The identification of Jun and Myc as in vitro substrates for the ERT protein kinase suggests that these proteins may be substrates for the ERT protein kinase in situ. One important test of this hypothesis is that the ERT protein kinase should be co-localized with its substrates. Jun and Myc are translated on cytoplasmic ribosomes and are subsequently transported into the nucleus. It is therefore possible that the cytosolic ERT protein kinase (4) may phosphorylate Jun and Myc prior to the entry of these proteins into the nucleus. Alternatively, it is possible that the ERT protein kinase may be located in both the nuclear and cytoplasmic compartments of the cell. As an initial approach to address this question we investigated the specific activity of the ERT protein kinase in cytosolic and nuclear extracts isolated from EGF-treated KB cells. The
specific activity of the ERT protein kinase was determined to be 5 ± 2 fmol/min/mg and 4 ± 1 fmol/min/mg in cytosolic and nuclear extracts, respectively (mean ± S.D., n = 3). The high specific activity of the ERT protein kinase in nuclear extracts compared with cytosolic extracts suggests that this enzyme may be located in the nucleus as well as the cytoplasm. However, two caveats must be applied to this conclusion. 1) An endogenous inhibitor of the ERT protein kinase (4) makes activity measurements an unreliable method for quantitating the level of enzyme expression. 2) There may be significant cross-contamination between the isolated cytosolic and nuclear fractions. A rigorous analysis of the subcellular localization of the ERT protein kinase will require the preparation of specific immunological reagents. These studies are currently in progress in this laboratory.

**DISCUSSION**

The purified ERT protein kinase has a restricted substrate specificity (4). The structural determinants that are employed by this enzyme for substrate recognition have not been defined. For many protein kinases it has been established that a critical factor for the specificity of phosphorylation is the primary sequence of substrate proteins (23). We therefore examined the primary sequence of the EGF receptor surrounding the phosphorylation site, Thr<sup>629</sup>. The distinctive feature of this phosphorylation site is the proximity of two proline residues: Val-Glu-Pro-Leu-Thr<sup>629</sup>-Pro-Ser-Gly. However, no rigorous analysis of this sequence can be achieved by visual inspection. Thus, an alternative approach was required to identify the amino acid residues that may be important for substrate recognition by the ERT protein kinase.

One strategy that has previously been successfully used to identify critical amino acid residues is the comparison of the sequences of proteins that have similar functions. The potential application of this approach to the ERT protein kinase is limited, because the EGF receptor is the only substrate that has been characterized (1–6). A major goal for the study described here was therefore the identification of additional protein substrates. We report here that the protein products of the proto-oncogenes c-jun and c-myc are substrates for phosphorylation by the ERT protein kinase.

**Phosphorylation of Myc Ser<sup>62</sup> in intact cells.** COS-1 cells were used for the transient expression of GAL4/Myc fusion proteins. The cells were labeled with [<sup>35</sup>S]methionine (panel A) or with [<sup>32</sup>P]phosphate (panel B) for 18 h. The cells were then incubated for 5 min at 37 °C with 10 nM EGF. The GAL4 proteins were isolated by immunoprecipitation and polyacrylamide gel electrophoresis. The gels were analyzed by fluorography (panel A) or by autoradiography (panel B). Lane 1, GAL4/Myc (1–147); lane 2, GAL4/Myc; lane 3, GAL4/Ala<sup>62</sup>Myc; lane 4, GAL4/Ala<sup>62</sup>Myc. Panel C shows the results of [<sup>32</sup>P]phosphoamino acid analysis. Tryptic [<sup>32</sup>P]phosphopeptide mapping of the GAL4/Myc fusion proteins is presented in panel D. The electrophoretic dimension (cathode at right) and the chromatography dimension are shown. The origin is marked with a cross at the lower left corner of each peptide map.

**Fig. 6. Phosphorylation of Myc Ser<sup>62</sup> in intact cells.** COS-1 cells were used for the transient expression of GAL4/Myc fusion proteins. The cells were labeled with [<sup>35</sup>S]methionine (panel A) or with [<sup>32</sup>P]phosphate (panel B) for 18 h. The cells were then incubated for 5 min at 37 °C with 10 nM EGF. The GAL4 proteins were isolated by immunoprecipitation and polyacrylamide gel electrophoresis. The gels were analyzed by fluorography (panel A) or by autoradiography (panel B). Lane 1, GAL4 (1–147); lane 2, GAL4/Myc; lane 3, GAL4/Ala<sup>62</sup>Myc; lane 4, GAL4/Ala<sup>62</sup>Myc. Panel C shows the results of [<sup>32</sup>P]phosphoamino acid analysis. Tryptic [<sup>32</sup>P]phosphopeptide mapping of the GAL4/Myc fusion proteins is presented in panel D. The electrophoretic dimension (cathode at right) and the chromatography dimension are shown. The origin is marked with a cross at the lower left corner of each peptide map.

**Fig. 7. Alignment of primary sequences of Jun family proteins.** The alignment of homologous amino acid sequences of human, mouse, rat, chicken, and viral Jun family proteins is presented. The alignment with the homologous sequence of the yeast transcription factor GCN4 is also shown. Previous studies have demonstrated that GCN4 is structurally and functionally homologous to the Jun family proteins (48, 49). A gap was introduced to allow the optimal alignment of the sequences. The asterisk indicates the serine residue in the rat c-jun protein (Ser<sup>246</sup>) that is phosphorylated by the ERT protein kinase.

**Reference**

- Human c-jun
- Mouse c-jun
- Rat c-jun
- Chicken c-jun
- ASV17 c-jun
- Mouse junB
- Mouse junD
- Yeast GCN4

| Reference | Sequence |
|-----------|----------|
| Human c-jun | PEMPGCE TPLPI DME |
| Mouse c-jun | PEMPGCE TPLPI DME |
| Rat c-jun | PEMPGCE TPLPI DME |
| Chicken c-jun | PEMPGCE TPLPI DME |
| ASV17 c-jun | PEMPGCE TPLPI DME |
| Mouse junB | PEMPGCE TPLPI DME |
| Mouse junD | PEMPGCE TPLPI DME |
| Yeast GCN4 | VAYKRQKPI |

fig. 7
Fig. 8. Alignment of primary sequences of Myc family proteins. The alignment of homologous amino acid sequences of human, mouse, rat, chicken, and viral Myc family proteins is presented. The asterisk indicates the amino acid residue in the human c-myc protein (Ser58) that is phosphorylated by the ERT protein kinase.

Fig. 9. Pro-Leu-Ser/Thr-Pro is a consensus primary sequence for substrate phosphorylation by the ERT protein kinase. The primary sequences surrounding the phosphorylation sites located in the EGF receptor (Thr35), Myc (Ser62), and Jun (Ser62) were aligned. A consensus sequence surrounding the phosphorylation sites is presented.

accounted for by the ERT protein kinase (or by another MAP2-related protein kinase). A possible role for glycogen synthase kinase-3 has also been proposed (20). Further work will be required to rigorously document the identity of the physiologically relevant Jun protein kinase.

Identification of a Consensus Primary Sequence for Substrate Phosphorylation by the ERT Protein Kinase—The site of in vivo phosphorylation of Myc by the ERT protein kinase was identified as Ser62 (Figs. 3 and 4). It is not known if Ser62 represents a site of phosphorylation of Myc in vivo. To address this question we constructed a GAL4/Myc fusion protein that has previously been demonstrated to be functional as a transcriptional activator (22). It was observed that Ser62 was the major site of phosphorylation of the GAL4/Myc fusion protein in COS-1 cells (Fig. 5). This phosphorylation was blocked by substitution of Ser62 with an alanine residue (Fig. 5). These data strongly implicate Ser62 as a site of Myc phosphorylation in vivo. It is possible that the in vivo phosphorylation of Ser62 may be accounted for by the ERT protein kinase, but for other protein kinases (e.g. the MAP2 protein kinase) cannot be excluded by the data reported here.

The physiological significance of the phosphorylation of Myc at Ser62 is not understood. The phosphorylation site is located in an amino-terminal region of the c-myc protein that is highly conserved between members of the Myc family (Fig. 8). Deletion studies have established that this amino-terminal region of Myc is required for neoplastic transformation (26) and is functional as a transcriptional activator (22). It is therefore possible that phosphorylation of Ser62 represents a mechanism of regulation of Myc function. Additional work will be required to test this hypothesis.

Identification of a Consensus Primary Sequence for Substrate Phosphorylation by the ERT Protein Kinase—Align-

One similarity between the sequences flanking the Pro-Leu-Ser/Thr-Pro consensus located in the EGF receptor, Myc, and Jun is that a glutamic acid residue is located close to the NH$_2$ terminus of the consensus sequence (Fig. 9). It is possible that this residue is important for substrate recognition, but the location of this residue is not highly conserved. Further work will be required to establish the significance of this observation.
2) Some of the potential substrates exhibit overlapping specificity with the cdc2 protein kinase. One example of overlapping substrate specificity is the Myc phosphorylation site Ser^{93} (Fig. 8). The sequence surrounding this site is Pro-Leu-Ser^{93}-Pro-Ser-Arg and corresponds to the consensus sequence for phosphorylation by the ERT protein kinase (Pro-Leu-Ser-Pro, Fig. 9) and the cdc2 protein kinase (Ser-Pro-X-Arg/Lys, Ref. 34). This observation is interesting because these protein kinases are active at different stages of the cell cycle. The cdc2 protein kinase is active during mitosis (34), but the ERT protein kinase is rapidly and transiently stimulated in nonmitotic cells by the addition of growth factors (2, 6). Thus, the phosphorylation state of Myc at Ser^{95} may be regulated by both cdc2 and ERT kinases during G1 phases of the cell cycle and by the cdc2 protein kinase during mitosis. Further studies are required to test this hypothesis, but evidence for the positive interphase phosphorylation of some cdc2 substrates has been obtained from studies of nuclear lamins (35-37).

Nuclear lamins are phosphorylated by the cdc2 protein kinase at several sites including Ser^{95} and Ser^{263} (35). Mitogen studies have established that both Ser^{95} and Ser^{263} are required for nuclear envelope breakdown during mitosis (36). Ser^{263} is specifically phosphorylated in response to mitogen-promoting factor (a complex that contains the cdc2 protein kinase), but Ser^{95} is phosphorylated during both mitosis and interphase (35). Examination of the primary sequence surrounding the mitosis-specific site, Ser^{263} (30, 31), indicates that it does not correspond to the consensus sequence identified for the ERT protein kinase (Fig. 8). In contrast, Ser^{95} is located within the primary sequence Pro-Leu-Ser-Pro-Thr-Arg, which contains the consensus for the cdc2 protein kinase (Ser-Pro-X-Arg/Lys, Ref. 34). As the cdc2 protein kinase is only activated during mitosis (34), it is unlikely that this enzyme can account for the interphase phosphorylation of nuclear lamins at Ser^{95}. A different protein kinase may therefore account for the phosphorylation of Ser^{95} observed during interphase (35). The ERT protein kinase represents a candidate enzyme that may account for this activity. This proposal requires a speculative hypothesis that suggests that the ERT protein kinase may exhibit some cdc2-like activity at early stages of the cell cycle. A rigorous test of this hypothesis is warranted.

Conclusions—The ERT protein kinase is a MAP2-related protein kinase that is markedly and rapidly stimulated by growth factors (2, 4, 6). Substrates phosphorylated by this enzyme include the nuclear proto-oncogene products Myc (Ser^{95}) and Jun (Ser^{263}). A consensus primary sequence for substrate phosphorylation was identified as Pro-Leu-Ser-Pro-Thr-Pro. Proteins containing this consensus sequence represent potential in vivo substrates for the ERT protein kinase. The phosphorylation of regulatory proteins such as Myc and Jun by the ERT protein kinase may represent an important pathway of signal transduction. A goal for further studies will be to test this hypothesis and to define the physiological significance of the phosphorylation during signal transduction.

Acknowledgments—We thank Dr. M. R. Green for providing GAL4 antisera and the plasmid pG4.24. We greatly appreciate the excellent secretarial assistance of Kim Gregoire.

Note Added in Proof—We have observed that the ERT protein kinase substrates Myc Ser^{95} and Jun Ser^{263} are also phosphorylated by the purified MAP2 protein kinase. It is therefore likely that Myc and Jun are phosphorylated by several members of the MAP2 family of protein kinases. Recently, it has been reported that the MAP2 kinase phosphorylates myelin basic protein at Thr^{27} within the sequence Pro-Arg-Thr^{27}-Pro (Erickson, A. K., Payne, M. D., Martino, P. A., Rossomando, A. J., Shabanowitz, J., Weber, M. J., Hunt, D. F. & Sturgill, T. W. (1990) J. Biol. Chem. 265, 19728-19735). Myelin basic protein is also phosphorylated at Thr^{27} by the ERT protein kinase (Ref. 4). Substrates that have been identified for MAP2 and MAP2-related kinases can therefore be represented by the consensus sequence Pro-Leu(Ser)/Thr-Pro. Further work will be required to establish the precise substrate specificity of these protein kinases.

REFERENCES
1. Heisermann, G. J. & Gill, G. N. (1988) J. Biol. Chem. 263, 13152-13158
2. Countaway, J. L., Northwood, I. C. & Davis, R. J. (1989) J. Biol. Chem. 264, 10828-10835
3. Countaway, J. L., McQuilkin, P., Gironis, N. & Davis, R. J. (1990) J. Biol. Chem. 265, 3407-3416
4. Northwood, I. C., Gonzalez, F. A., Wartmann, M., Raden, D. L. & Davis, R. J. (1991) J. Biol. Chem. 266, 15266-15276
5. Heisermann, G. J., Wiley, H. S., Walsh, B. J., Ingraham, H. A., Fiol, C. J. & Gill, G. N. (1990) J. Biol. Chem. 265, 12820-12827
6. Northwood, I. C. & Davis, R. J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6107-6111
7. Lee, K. A. W., Bindereif, A. & Green, M. R. (1988) Genet. Anal. Tech. 5, 22-31
8. Abate, C., Luk, D. & Curran, T. (1990) Cell Growth & Differ. 1, 455-462
9. Abate, C., Luk, D., Gentz, R., Rauscher, F. J. & Curran, T. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1092-1096
10. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. & Penae, L. R. (1989) Gene (Amst.) 77, 51-59
11. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463-5467
12. Koland, J. G., O’Brien, K. M. & Cerione, R. A. (1990) Biochem. Biophys. Res. Commun. 170, 90-100
13. Sadowski, L. M., Mor, J., Trizenberg, S. & Ptasnne, M. (1988) Nature 335, 563-564
14. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
15. Laemmli, U. K. (1970) Nature 227, 680-685
16. Hunter, T. & Sefton, B. M. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 1311-1315
17. Davis, R. J. & Czech, M. P. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 1974-1978
18. Beemon, K. & Hunter, T. (1978) J. Virol. 28, 551-566
19. Davis, R. J., Johnson, G. L., Kelleher, D. J., Anderson, J. K., Mole, J. E. & Czech, M. P. (1986) J. Biol. Chem. 261, 9034-9041
20. Boyle, W. J., Smeal, T., Defize, L. H. K., Angel, J. R., Karin, M. & Hunter, T. (1991) Cell 64, 573-584
21. Luscher, B., Kuenzel, E. A., Krebs, E. G. & Eisenman, R. N. (1989) EMBO J. 8, 1111-1119
22. Kato, G. J., Barrett, J., Villa-Garcia, M. & Dang, C. V. (1990) Mol. Cell. Biol. 10, 5914-5920
23. Edelman, A. M., Blumenthal, D. K. & Krebs, E. G. (1987) Annu. Rev. Biochem. 56, 567-613
24. Koppola, T. K. & Curran, T. (1991) Curr. Biol. (Lond.) 1, 71-79
25. Nishimura, T. & Vogt, P. K. (1988) Oncogene 3, 659-663
26. Stone, J., Delange, T., Ramsay, G., Jakobovits, E. & Bishop, J. M. (1987) Mol. Cell. Biol. 7, 1697-1709
27. Berleth, T., Burri, M., Thomas, G., Bopp, D., Richstein, S., Frigerio, G. & Noll, M. (1988) EMBO J. 7, 1749-1756
28. Yokochi, M. & Byers, B. (1987) J. Mol. Biol. 195, 233-245
29. Russell, P. & Nurse, P. (1987) Cell 49, 559-567
30. Fisher, D. Z., Chaudhary, N. & Blobel, G. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6456-6454
31. Meenon, F. D., Kirschner, M. W. & Caput, D. (1986) Nature 319, 463-468
32. Colby, W. W., Chen, E. Y., Smith, D. H. & Levinson, A. D. (1983) Nature 301, 722-725
33. Hattori, K., Angel, P., Le Beau, M. M. & Karin, M. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 9148-9152
34. Moreno, S. & Nurse, P. (1988) Cell 51, 549-551
35. Ward, G. E. & Kirschner, M. W. (1990) Cell 61, 561-577
36. Heald, R. & McKeon, F. (1990) Cell 61, 579-589
ERT Protein Kinase Substrate Specificity

37. Peter, M., Nakagawa, J., Dorée, M., Labbé, J. C., & Nigg, E. A. (1990) *Cell* **61**, 591-602
38. Coussens, L., Yang-Feng, T. L., Liao, Y. C., Chen, E., Gray, A., McGrath, J., Seeburg, P. H., Libermann, T. A., Schlessinger, J., Francke, U., Levinson, A. & Ullrich, A. (1985) *Science* **230**, 1132-1139
39. Hunter, T., Ling, N. & Cooper, J. A. (1984) *Nature* **311**, 480-483
40. Kitabayashi, I., Saka, F., Gachelin, G. & Yokoyama, K. (1990) *Nucleic Acids Res.* **18**, 3400
41. Ryseck, R. P., Hirai, S. I., Yaniv, M. & Bravo, R. (1988) *Nature* **334**, 535-537
42. Ryder, K. & Nathans, D. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 8464-8467
43. Lamph, W. W., Wamsley, P., Sassone-Corsi, P. & Verma, I. (1988) *Nature* **334**, 629-631
44. Maki, Y., Bos, T. J., David, C., Starbuck, M. & Vogt, P. K. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 2848-2852
45. Ryder, K., Lau, L. F. & Nathans, D. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 1487-1491
46. Hirai, S. I., Ryseck, R. P., Mechta, F., Bravo, R. & Yaniv, M. (1989) *EMBO J.* **8**, 1433-1439
47. Hope, I. A. & Struhl, K. (1986) *Cell* **46**, 885-894
48. Vogt, P. K., Bos, T. J. & Doolittle, R. F. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 3316-3319
49. Struhl, K. (1987) *Cell* **50**, 841-846
50. Bernard, O., Cory, S., Gerondakis, S., Webb, E. & Adams, J. M. (1983) *EMBO J.* **2**, 2375-2383
51. Hayashi, K., Makino, R., Kawamura, H., Arisawa, A. & Yoneda, E. (1987) *Nucleic Acids Res.* **15**, 6419-6436
52. Watson, D., Psallidopoulos, M., Samuel, K., Dalla-Favera, R. & Papas, T. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80**, 3642-3645
53. Kan, N. C., Flordellis, C. S., Mark, G. E., Duesberg, P. H. & Papas, T. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 3000-3004
54. Reddy, E. P., Reynolds, R. K., Watson, D. K., Schulz, R. A., Lautenberger, J. & Papas, T. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80**, 2500-2504
55. Legouy, E., DePinho, R., Zimmerman, K., Collum, R., Vancopoulou, G., Mitsock, L., Kriz, R. & Alt, F. W. (1987) *EMBO J.* **6**, 3359-3366
56. Taya, Y., Mizusawa, S. & Nishimura, S. (1986) *EMBO J.* **5**, 1215-1219
57. Sugiyama, A., Kume, A., Nemoto, K., Lee, S. Y., Agami, Y., Nemoto, F., Nishimura, S. & Kuchino, Y. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 9144-9148