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# Protein kinase C activation potently down-regulates the expression of its major substrate, 80K, in Swiss 3T3 cells

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**The amino acid sequence of 80K, the major acidic protein kinase C (PKC) substrate of Swiss 3T3 fibroblasts, was deduced from a cDNA nucleotide sequence. Overall, 25% of the predicted amino acid sequence is supported by direct protein sequence data. Southern blot analysis suggests that the mouse genome contains a single copy of this gene. Two 80K mRNA species, a major band of 2.25 kb and a minor band of 3.9 kb, were detected by Northern blot analysis. Stimulation of PKC by biologically active phorbol esters, including phorbol-12, 13-dibutyrate (PDB), reduced the steady state level of 80K mRNA to 8.8% of control within 5–7 h. This effect was dose-dependent, and was abolished by prior depletion of PKC. The PDB-induced down-regulation of 80K mRNA levels was transient, and recovery coincided with the disappearance of PKC activity. A similar transient decrease in 80K mRNA levels was also demonstrated in tertiary cultures of mouse embryo fibroblasts. The down-regulation of 80K mRNA levels was completely abolished by actinomycin D, cycloheximide or anisomycin if added up to 30 min after PDB addition. Since the rate of transcription of the 80K gene was unaltered by PDB treatment, we concluded that the PKC-induced down-regulation of 80K mRNA is mediated by a post-transcriptional mechanism. In addition, PDB transiently decreased the level of 80K protein within 14–18 h, thus reflecting the effects of this phorbol ester on mRNA expression.**

*Key words:* cellular signalling/molecular cloning/mRNA expression/phorbol ester/protein kinase C substrate

## Introduction

Protein kinase C (PKC) comprises a family of closely related enzymes that are activated by the second messenger diacylglycerol and serve as the target for tumour-promoting phorbol esters (Nishizuka, 1986, 1988). These enzymes are involved in the signal transduction of many short-term responses including secretion of hormones and enzymes, neurotransmitter release, alterations in membrane ionic conductance and muscle contraction (Kikkawa and Nishizuka, 1986). Furthermore, PKC activation has been implicated in the regulation of long-term responses such as gene expression and cell proliferation (Rozengurt and Sinnett-Smith, 1988). Since the mechanisms through which PKC mediates these cellular responses remain largely unknown, it is essential to characterize the physiological substrates of this kinase family.

An acidic protein that migrates with an apparent molecular mass of 80 kDa in SDS–polyacrylamide gels, termed 80K, has been identified as a major and specific substrate for PKC in quiescent mouse 3T3 fibroblasts (Rozengurt *et al.*, 1983, 1984; Rodriguez-Pena and Rozengurt, 1985, 1986a; Tsuda *et al.*, 1985). This protein is reversibly phosphorylated by a variety of growth factors and pharmacological agents, including bombesin, vasopressin, bradykinin and phorbol esters (Rozengurt *et al.*, 1983; Rodriguez-Pena and Rozengurt, 1985, 1986b; Isacke *et al.*, 1986; Rodriguez-Pena *et al.*, 1986; Zachary *et al.*, 1986; Erusalimsky *et al.*, 1988; Erusalimsky and Rozengurt, 1989; Issandou and Rozengurt, 1990) all of which are known to stimulate PKC. A reduction in the level and phosphorylation of 80K has been shown to accompany both a loss of mitogenic responsiveness to phorbol esters in 3T3 cell variants (Beimann and Erickson, 1990), and conversion to the transformed phenotype in *ras*, *src* or *fms* transformed NIH3T3 fibroblasts (Wolfman *et al.*, 1987). These findings raise the possibility that 80K is involved in mitogenic signal transduction and transformation by certain oncogenes, and suggest that this protein may be regulated through alterations in both phosphorylation and expression.

We have recently reported the purification and partial amino acid sequence of 80K from Swiss 3T3 fibroblasts (Brooks *et al.*, 1990). Acidic cellular 65–78 kDa PKC substrates that appear to be related to the protein found in Swiss 3T3 fibroblasts have also been identified in a wide variety of mammalian cultured cells (Rozengurt *et al.*, 1983; Coughlin *et al.*, 1985; Rodriguez-Pena and Rozengurt, 1985; Blackshear *et al.*, 1985, 1986; Hornbeck and Paul, 1986; Aderem *et al.*, 1988; Hirai and Shimizu, 1989; Hornbeck *et al.*, 1989) as well as in brain (Blackshear *et al.*, 1986; Albert *et al.*, 1986; Morris and Rozengurt, 1988; Graff *et al.*, 1989a; Erusalimsky *et al.*, 1991) and other tissues (Albert *et al.*, 1986; Blackshear *et al.*, 1986). Comparison of the amino acid sequences deduced from the cDNA clones encoding both rat brain 80K (Erusalimsky *et al.*, 1991) and the bovine brain 87 kDa PKC substrate, termed MARCKS (Stumpo *et al.*, 1989), has revealed that they are related but not identical. Furthermore, a cDNA encoding an acidic 80 kDa PKC substrate from epidermal carcinoma cells predicts an entirely different amino acid sequence (Sakai *et al.*, 1989). These findings indicate that the acidic 65–87 kDa proteins may be more diverse than previously thought. Thus, in order to assess the functional implications of this diversity and to analyse the long-term regulation of this protein, it is necessary to define the structure of each individual substrate in detail.

The non-tumorigenic murine Swiss 3T3 fibroblast cell line has proved to be a useful model system for identifying both extracellular factors that modulate cell growth and for elucidating the early signals that lead to mitogenesis

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(Rozengurt, 1986). In the present study, we have deduced the complete amino acid sequence of the Swiss 3T3 fibroblast 80K protein from its cDNA sequence and have investigated the role of PKC in the regulation of 80K mRNA and protein expression. Surprisingly, we find that activation of PKC leads to a striking down-regulation of 80K mRNA levels in Swiss 3T3 fibroblasts through a post-transcriptional mechanism.

## Results

### *cDNA sequence of Swiss 3T3 fibroblast 80K*

A 71mer oligonucleotide probe, spanning a region highly conserved between the mouse fibroblast (Brooks *et al.*, 1990) and rat brain 80K proteins (Erusalimsky *et al.*, 1991), was synthesized and used to screen a Swiss 3T3 fibroblast λZAPII cDNA library. A total of 19 positive clones were isolated, 14 of which were shown to contain homologous sequences by restriction mapping and partial DNA sequencing. The complete DNA sequence encoding the 80K protein was determined by sequencing three overlapping clones and is 1900 bp in length, including a putative poly(A) tail of 15 bases (Figure 1). At the 5′ end, 282 nucleotides precede an ATG codon, which is present in the consensus sequence for translation initiation (Kozak, 1987). After the translation start site, there is a single open reading frame, which predicts a protein of 309 amino acid residues. Overall, 25% of the deduced amino acid sequence is supported by direct protein sequence data (Brooks *et al.*, 1990), allowing the unequivocal identification of the cDNA clones. At the 3′ end of the coding region, an in-frame stop codon at position 1210 is followed by 688 bp of untranslated sequence. A consensus polyadenylation signal (AATAAA) is present 25 bp upstream from the putative poly(A) tail.

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     TTT TAA AAA AAC TAC ACT TGG GCT CCT TTT TTG TGC TCG ACT TTT CCA CCT TTT
     TCC CTC CTT CCT GCG CTG CTG CTT TTT TGA TCT CTT CGA CTA AAA ATT TTT TAT   108
     CCG GAG TAT TTA ATC GGG TCT CTT CTG TCC TCC TCG CCA CCC CCA CCC CCT CCC
     TCC GGT GTG TGT GCC GCC GCC GCT GTT GCT GCT GCT GCT GCT CGC CCC GTC GTT   216
     ACA CCA ACC GCC GGC TCT TTG TTT CCT CTC TTG GAT CTG TTG AGT TTC TTT GTT
     GAA GAA GCC AGC ATG GGT GCC CAG TTC TCC AAG ACC GCA GCG AAG GGA GAA GCC   324
  1                  M   G   A   Q   F   S   K   T   A   A   K   G   E   A

     ACC GCC GAG AGG CCC GGG GAG GCG GCT GTG GCC TCG TCG CCT TCC AAA GCA AAT   378
 15  T   A   E   R   P   G   E   A   A   V   A   S   S   P   S   K   A   N

     GGG CAG GAG AAT GGC CAC GTA AAA GTG AAC GGG GAC GCG TCT CCC GCC GCC GCC   432
 33  G   Q   E   N   G   H   V   K   V   N   G   D   A   S   P   A   A   A

     GAG CCG GGC GCC AAG GAG GAG CTG CAA GCC AAC GGC AGC GCC CCG GCC GCC GAC   486
 51  E   P   G   A   K   E   E   L   Q   A   N   G   S   A   P   A   A   D

     AAG GAG GAG CCC GCG AGC GGC AGT GCC GCG ACC CCC GCC GCG GCC GAA AAG GAT   540
 69  K   E   E   P   A   S   G   S   A   A   T   P   A   A   A   E   K   D

     GAG GCT GCC GCG GCC ACC GAG CCG GGC ACC GGC ACG GCC GAC AAG GAG GCT GCG   594
 87  E   A   A   A   A   T   E   P   G   T   G   T   A   D   K   E   A   A

     GAG GCC GAG CCC GCC GAG CCC AGC TCC CCG GCC GCC GAG GCC GAG GGC GCG TCC   648
105  E   A   E   P   A   E   P   S   S   P   A   A   E   A   E   G   A   S

     GCC TCC TCC ACG TCG TCG CCC AAG GCG GAG GAC GGG GCC GCG CCG TCG CCC AGC   702
123  A   S   S   T   S   S   P   K   A   E   D   G   A   A   P   S   P   S

     AGC GAG ACC CCG AAA AAA AAA AAG AAG CGC TTT TCC TTC AAG AAG TCC TTC AAG   756
141  S   E   T   P   K   K   K   K   K   R   F   S   F   K   K   S   F   K

     CTG AGC GGC TTC TCC TTC AAG AAG AGC AAG AAG GAG TCG GGC GAG GGC GCT GAA   810
159  L   S   G   F   S   F   K   K   S   K   K   E   S   G   E   G   A   E

     GCA GAG GGA GCG ACC GCG GAA GGC GCC AAG GAC GAG GCT GCA GCC GCA GCG GGC   864
177  A   E   G   A   T   A   E   G   A   K   D   E   A   A   A   A   A   G

     GGC GAG GGG GCC GCG GCC CCC GGG GAG CAG GCA GGC GGG GCG GGC GCC GAG GGC   918
195  G   E   G   A   A   A   P   G   E   Q   A   G   G   A   G   A   E   G

     GCG GCG GGC GGA GAG CCC CGG GAG GCC GAG GCG GCG GAG CCC GAG CAG CCG GAG   972
213  A   A   G   G   E   P   R   E   A   E   A   A   E   P   E   Q   P   E

     CAG CCG GAG CAG CCC GCG GCG GAG GAG CCG CAG GCG GAG GAG CAG TCG GAG GCG  1026
231  Q   P   E   Q   P   A   A   E   E   P   Q   A   E   E   Q   S   E   A

     GCG GGG GAG AAG GCG GAG GAG CCC GCG CCC GGC GCC ACC GCG GGC GAT GCG TCC  1080
249  A   G   E   K   A   E   E   P   A   P   G   A   T   A   G   D   A   S

     TCC GCC GCA GGG CCT GAG CAG GAG GCG CCC GCT GCC ACC GAC GAG GCC GCG GCG  1134
267  S   A   A   G   P   E   Q   E   A   P   A   A   T   D   E   A   A   A

     TCC GCA GCC CCC GCC GCG TCG CCG GAG CCG CAG CCC GAG TGC AGT CCG GAG GCG  1188
285  S   A   A   P   A   A   S   P   E   P   Q   P   E   C   S   P   E   A

     CCC CCC GCG CCA ACG GCC GAG TAA GCT CCA GAG CCC TCA CGC AAT TCA AGA ACT  1242
302  P   P   A   P   T   A   E

     TTT CCC CCC AGT TTG TTT GTT GGA GTG GTG CCA GGT ACT GGT TTT GGA GAA CTT  1296
     GTC TAC AAC CAG GGA TTG ATT TTA AAG ATT TTT TTT TAA ATT TCA CAT TTT TTT
     TAA GCA GCA AAT TTT TTG TTT TTT GTT TTT TTT AAG CCC CCT TCC CCA CAG ATC  1404
     CCA TCT CAG ATA GTT GTT TCC ACC ATT CCG ACA GGC CGA GGA CGT GTT AGA CAG
     CTT CCT CTG CCT TCT TTC TTT ACT TTT ACT TTT TTT TTT TTT TTT TGG CAT CAG  1512
     TAT TAA TGT TTT TTG CAT ACT TTG CAT CTT TAT TAA AAA AGT GTA AAC TTT CTT
     TGT CAG ATC TAT AGA CAT ACC CAT ATA TGA AGG AGA TGG GTG GGT CAA AAG GAA  1620
     TAA CAA ATG AAG TGA TAG GGG CCA CTA TGG GAA ATT GAA GCA GTG CAT AAC ATT
     GCC AAG ATA ATA TGC CAC TAA AAT GGT GGT GGG TGT AAA GGC TTA GGG TTC TTG  1728
     TCC TTT CTT TCT TTC TTT CTT TCT TTC TTT CTT TCT TTC TTT CTT TTT TTT TTT
     AAA GAA AAA TTA TTA CGA TGT ATT TTG TGA GGC AGG TTT ACA ACA CTA CAC GTT  1836
     TTG AAT AAG AAG GAA AGA GAA AAA AAT AAA AAC ACC AAT ACC CAG ATT TAA AAA
     AAA AAA AAA A                                                            1900
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**Fig. 1.** Nucleotide sequence of the 80K cDNA clone and the deduced primary structure of the protein. The complete cDNA sequence was obtained by sequencing three overlapping clones [p803.3 (97–1572 bp), p809.1 (1–1233 bp) and p8017.1 (391–1900 bp)]. Numbers on the left indicate the position of the amino acids, and on the right, positions of the nucleotides. Peptide sequences confirmed by Edman degradation are underlined. In the 3′ untranslated region, the polyadenylation signal is underlined.

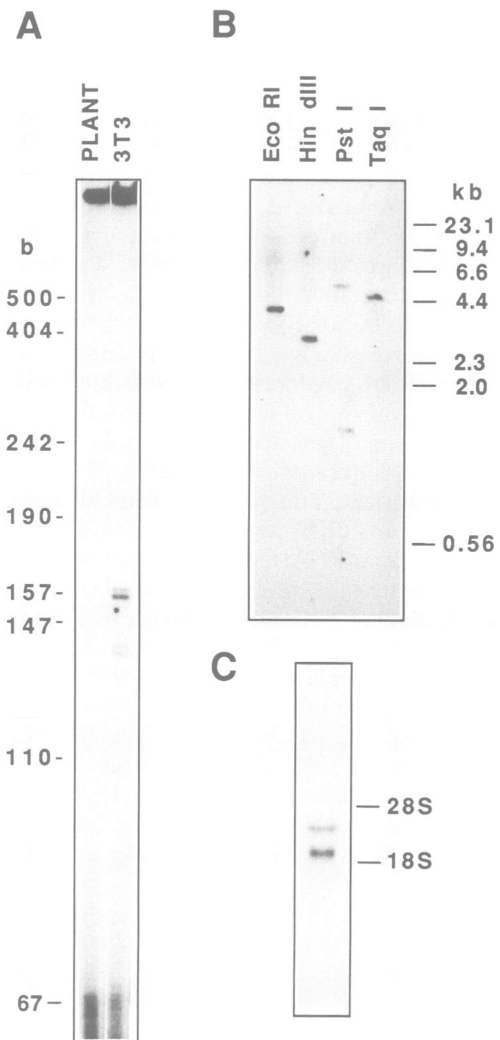


**Fig. 2.** **(A)** Primer extension analysis. A 5′ end-labelled oligonucleotide probe complementary to the region between bases 20–50 of the 80K cDNA clone was hybridized to either 3T3 or tobacco plant RNA, and the extension products were analysed on a 6% sequencing gel. The positions of fragments of pBluescript SK(−) digested with *Hpa*II and labelled by fill-in reaction with [α-$^{32}$P]dCTP is shown on the left. **(B)** Genomic Southern blot analysis. Genomic DNA from Swiss 3T3 fibroblasts was digested with *Eco*RI, *Hin*dIII, *Pst*I or *Taq*I and analysed by Southern blotting using a 843 bp *Bal*I–*Eco*RI cDNA fragment of p809.1 as a probe. The positions of the λDNA/*Hin*dIII fragments are shown on the right. **(C)** Northern blot analysis. Total cellular RNA (10 μg) isolated from Swiss 3T3 fibroblasts was analysed by Northern blotting using the 843 bp *Bal*I–*Eco*RI probe. The positions of the 28S and 18S ribosomal RNAs are shown on the right.

The extent of the 5′ untranslated region of the 80K mRNA was determined by primer extension analysis of total RNA from Swiss 3T3 fibroblasts. The most prominent extension products were a doublet 157−159 bases upstream of the 5′ end of the primer, which corresponds to a position 107−109 bp from the 5′ end of the cDNA clone (Figure 2A). Therefore, the complete 5′ untranslated region is 389−391 bp in length. No extension products were detected when RNA from a totally unrelated species (tobacco plant) was used as the template, which confirms the specificity of the primer.

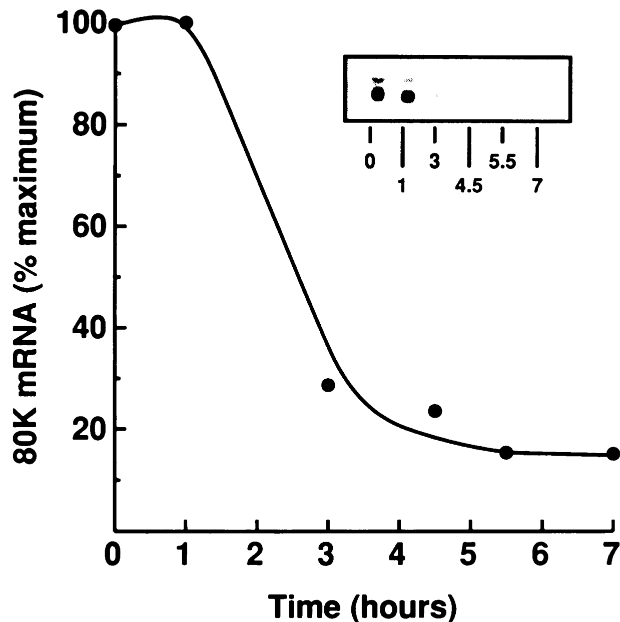


**Fig. 3.** Time-course of PDB-induced down-regulation of 80K mRNA. Confluent, quiescent Swiss 3T3 fibroblasts in 90 mm dishes (4 × $10^6$ cells) were incubated with 800 nM PDB for various periods of time. Total cellular RNA was prepared from each sample and 10 μg was analysed by Northern blotting using the 843 bp *Bàl*I−*Eco*RI probe. Levels of 80K mRNA were quantified by densitometric scanning of the autoradiograph as described in Materials and methods.

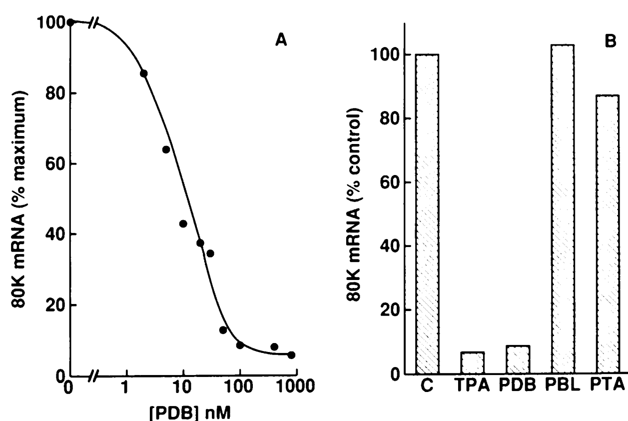


**Fig. 4.** (**A**) Dose dependence of PDB-induced down-regulation of 80K mRNA. Confluent, quiescent Swiss 3T3 fibroblasts in 90 mm dishes (4 × $10^6$ cells) were incubated with various concentrations of PDB for 5 h. Total cellular RNA was isolated and 80K mRNA levels analysed by Northern blotting as described in Materials and methods.
(**B**) Specificity of phorbol ester-induced down-regulation of 80K mRNA. Confluent, quiescent Swiss 3T3 fibroblasts in 90 mm dishes (4 × $10^6$ cells) were incubated with 800 nM PDB, 12-O-tetradecanoyl-phorbol 13-acetate (TPA), phorbol 12,13,20-triacetate (PTA) or phorbol (PBL) for 5 h. Total cellular RNA was isolated and 80K mRNA levels analysed by Northern blotting.

### *Northern and Southern blot analysis*

Genomic Southern blot analysis was performed on Swiss 3T3 fibroblast DNA digested with *Eco*RI, *Hin*dIII, *Pst*I and *Taq*I. When hybridized with a *Bal*I−*Eco*RI cDNA fragment of p809.1 (see legend to Figure 1), two bands were detected with *Pst*I and a single band with *Eco*RI, *Hin*dIII and *Taq*I (Figure 2B). The 80K cDNA sequence contains a unique *Pst*I site, but no sites for *Eco*RI, *Hin*dII or *Bam*HI. Therefore, the hybridization pattern is consistent with that predicted from the nucleotide sequence of 80K, and indicates that the mouse genome contains a single copy of this gene.

Two transcripts of 2.25 and 3.9 kb were detected on Northern blots of either total cellular RNA (Figure 2C) or poly(A)$^+$ RNA (data not shown) from Swiss 3T3 fibroblasts when hybridized with the 843 bp *Bal*I−*Eco*RI probe.

### *Primary structure analysis*

The Swiss 3T3 fibroblast 80K protein has a calculated molecular mass of 29 722 kDa and pI of 4.10. The protein shows homology with other acidic 65−87 kDa PKC substrates, including the rat brain 80K protein (Erusalimsky *et al.*, 1991) and bovine brain MARCKS (Stumpo *et al.*, 1989). The overall sequence identity between the mouse fibroblast 80K protein (Figure 1), and the rat and bovine brain proteins is 93% and 59% respectively.

Two regions of high conservation between these proteins are the amino terminus, which contains potential myristoylation and N-glycosylation sites, and the lysine-rich region in the central portion of the protein containing four of the six putative PKC phosphorylation sites that comprise a potential calmodulin-binding domain (Graff *et al.*, 1989b). In contrast, 80K does not exhibit any significant similarity to other PKC substrates such as GAP-43 (Karns *et al.*, 1987), p47 (Tyres *et al.*, 1988) or the human epidermal carcinoma 80K-H protein (Sakai *et al.*, 1989).

### *Effect of phorbol esters on 80K mRNA levels in Swiss 3T3 fibroblasts*

PKC has been shown to increase the expression of a number of genes, including c-*fos*, c-*myc* and tyrosine hydroxylase (Kelly *et al.*, 1983; Campisi *et al.*, 1984; Cochran *et al.*, 1984; Greenberg and Ziff, 1984; Kruijer *et al.*, 1984; Muller *et al.*, 1984; Kaibuchi *et al.*, 1986; Vyas *et al.*, 1990). All of these proteins are physiological substrates of PKC (Barber and Verma, 1987; Saksela *et al.*, 1989; Zigmond *et al.*, 1989). Consequently, it was of interest to determine if PKC could regulate the expression of the mRNA encoding the 80K protein. First, we investigated the effect of exposure of Swiss 3T3 fibroblasts to PDB on the levels of 80K mRNA by Northern blot analysis using the 843 bp *Bal*I−*Eco*RI cDNA fragment of p809.1 as a probe. Treatment with 800 nM PDB for various periods of time resulted in a dramatic decrease in 80K mRNA levels. After an initial lag period of 1 h, 80K mRNA declined rapidly to a minimum of 8.8 ± 5.6%($n$ = 8) of control after 5 h of incubation (Figure 3).

PDB decreased 80K mRNA levels in a dose-dependent manner. A half-maximal effect was achieved at 11 nM (Figure 4A), which is similar to the equilibrium dissociation

constant of [$^3$H]PDB binding to PKC in Swiss 3T3 cells (Collins and Rozengurt, 1982). The specificity of this response was investigated using the alcohol phorbol, and its derivative phorbol 12,13,20-triacetate, both of which are biologically inactive. Neither compound depleted 80K mRNA when measured after a period of 5 h in contrast to the biologically active esters, PDB and 12-O-tetradecanoyl-phorbol 13-acetate (TPA; Figure 4B).

If the decrease in 80K mRNA levels induced by the biologically active phorbol esters is mediated by PKC, then prior down-regulation of this enzyme should block the response. Therefore, cells were pre-treated with a saturating dose of PDB (800 nM) for 48 h to down-regulate PKC (Rodriguez-Pena and Rozengurt, 1984) and subsequently challenged with phorbol esters for 5 h. As shown in Figure 5A, the striking decrease in 80K mRNA in response to either PDB or TPA was completely abolished by prior down-regulation of PKC. Furthermore, the level of 80K mRNA in these pre-treated cells was restored to that of untreated cultures. Thus, it may be predicted that treatment with PDB over a long period should cause an initial decrease in levels of 80K mRNA, followed by a recovery due to down-regulation of PKC. Indeed, 80K mRNA levels decreased transiently, reaching a minimum after 5–7 h and recovered to ~80% of control after 24 h (Figure 5B). These results establish the involvement of PKC in the regulation of 80K mRNA expression in Swiss 3T3 fibroblasts.

### Effect of PDB on 80K mRNA levels in tertiary passage mouse embryo fibroblasts

To determine whether the PKC-mediated down-regulation of 80K mRNA can be reproduced in diploid cells, we analysed the effect of PDB treatment on 80K mRNA levels in quiescent cultures of tertiary mouse embryo fibroblasts. Previous reports have indicated that the 80K protein is more abundant in these cells compared to Swiss 3T3 fibroblasts (Rodriguez-Pena and Rozengurt, 1985). Consistent with this finding, Northern blot analysis revealed the presence of two transcripts in these cells of similar size to those of Swiss 3T3 fibroblasts, but expressed at significantly higher levels (Figure 6A). When these cells were treated with PDB for various periods of time, a transient decrease in 80K mRNA levels was detected, with similar kinetics to Swiss 3T3 fibroblasts (Figure 6B). Thus, the striking down-regulation of 80K mRNA levels by PDB is not confined to the Swiss 3T3 fibroblast cell line.

### Mechanism of regulation of 80K mRNA levels

The PKC-induced down-regulation of 80K mRNA could be mediated by a decrease in either its rate of transcription, RNA stability or both. If this effect is the result of reduced promoter activity, the 80K mRNA would be predicted to be unstable as the effect of PDB is evident within 3 h and maximal within 5–7 h. In order to determine the stability of 80K mRNA, quiescent cultures of Swiss 3T3 fibroblasts were treated with actinomycin D (4 $\mu$M) to block RNA synthesis, and at various times after addition (1, 3, 5, 7, 9 and 16 h), levels of 80K mRNA were analysed by Northern blotting. The apparent half-life was 11.5 h (data not shown). Therefore, this result suggests that the effect of PDB on 80K mRNA levels is not due to a decrease in its rate of transcription. To test this directly, run-off transcription assays were performed on nuclei isolated from

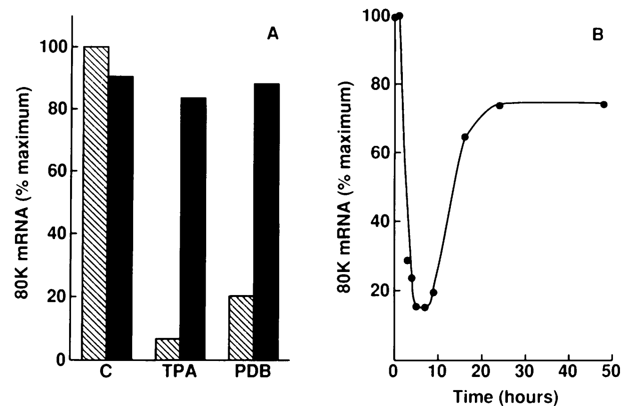


**Fig. 5.** (**A**) Effect of PKC depletion on the phorbol ester-induced decrease in 80K mRNA levels. Controls (hatched bars) or PDB pre-treated (solid bars) cultures of confluent, quiescent Swiss 3T3 fibroblasts were exposed to 800 nM PDB or TPA for 5 h. Total cellular RNA was isolated and 80K mRNA levels were analysed by Northern blotting as described in Materials and methods. (**B**) Extended time-course for PDB-induced down-regulation of 80K mRNA. Swiss 3T3 fibroblasts in 90 mm dishes (4 × $10^6$ cells) were incubated with 800 nM PDB for various periods of time. Total cellular RNA was isolated and levels of 80K mRNA were analysed by Northern blotting.

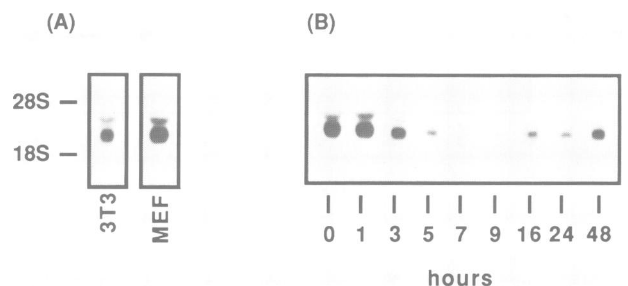


**Fig. 6.** (**A**) Northern blot analysis of RNA from Swiss 3T3 and tertiary passage mouse embryo fibroblasts. Total cellular RNA (10 $\mu$g) isolated from confluent, quiescent cultures of either Swiss 3T3 fibroblasts or tertiary passage mouse embryo fibroblasts (MEF) was analysed by Northern blotting as described in Materials and methods. (**B**) Time-course of PDB-induced down-regulation of 80K mRNA from tertiary passage mouse embryo fibroblasts. Confluent, quiescent tertiary passage mouse embryo fibroblasts in 90 mm dishes (4 × $10^6$ cells) were incubated with 800 nM PDB for various periods of time. Total cellular RNA was isolated and 80K mRNA levels analysed by Northern blotting.

quiescent Swiss 3T3 fibroblasts that had been exposed to PDB for 5 h. To establish the integrity of the isolated nuclei, the effect of PDB on c-*fos* gene transcription was analysed 15 min after exposure of cells to phorbol ester. Consistent with previous results obtained in 3T3 fibroblasts (Greenberg and Ziff, 1984), the transcription rate of c-*fos* increased transiently >23-fold after 15 min treatment with PDB, and returned to basal levels within 5 h. In contrast, there was little change in the rate of transcription of either the 18S subunit of ribosomal RNA or cytochrome C oxidase after phorbol ester addition. Importantly, the rate of transcription of the 80K gene remained unaltered after treatment of cells with PDB for 5 h (Figure 7), at which time the steady state level of 80K mRNA is at a minimum. Taken together, these data suggest that the effect of PKC on 80K mRNA expression is mediated through a post-transcriptional mechanism, probably involving a decrease in mRNA stability.

Since there is a lag period of 1 h before PDB has any effect

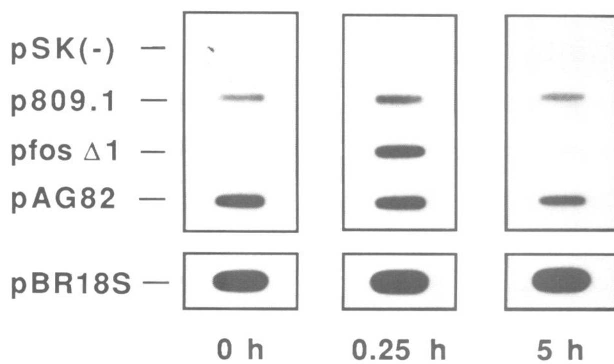


**Fig. 7.** Transcriptional analysis of the 80K gene after PDB treatment of Swiss 3T3 fibroblasts. Plasmids containing inserts encoding for 80K (p809.1), *c-fos* (pfosΔ1) cytochrome C oxidase (pAG92), the 18S subunit of ribosomal RNA (pBR18S) and the control vector pBluescript (SK−), (pSK−), were hybridized with $^{32}$P-labelled run-off transcripts from nuclei isolated from quiescent, confluent Swiss 3T3 fibroblasts that had been exposed to 800 nM PDB for either 15 min or 5 h as described in Materials and methods. Under these conditions, there was no hybridization to the pBluescript (SK−) vector. Filters were autoradiographed with Kodak X-Omat AR X-ray film for either 16 h (pBR18S) or 36 h [pSK(−), p809.1, pfosΔ1, pAG82] at −70°C.

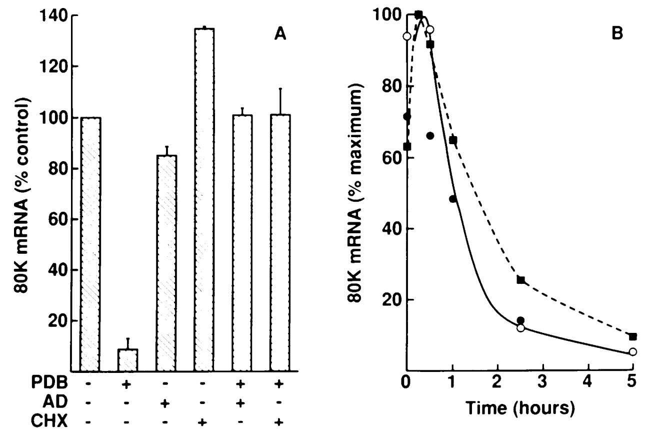


**Fig. 8.** Effect of RNA or protein synthesis inhibitors on PDB-induced down-regulation of 80K mRNA. (**A**) Confluent, quiescent Swiss 3T3 fibroblasts in 90 mm dishes (4 × $10^6$ cells) were incubated in the presence of the indicated combinations of PDB (800 nM), actinomycin D (4 μM) and cycloheximide (25 μM) for 5 h. Total cellular RNA was isolated and 80K mRNA levels analysed by Northern blotting as described. Data are expressed as mean ± standard error from two independent experiments. (**B**) Confluent, quiescent Swiss 3T3 fibroblasts in 90 mm dishes (4 × $10^6$ cells) were incubated with 800 nM PDB for 5 h, and then at various times during this incubation period, (■) actinomycin D (4 μM), (○) cycloheximide (25 μM) or (●) anisomycin (50 μM) were added. Total cellular RNA was isolated and 80K mRNA levels analysed by Northern blotting as described in Materials and methods. The single solid line represents the effect of the two protein synthesis inhibitors, cycloheximide and anisomycin.

on 80K mRNA levels, it is possible that PKC induces the synthesis of a protein which subsequently destabilizes 80K mRNA. To investigate this possibility, we analysed the effect of blocking both RNA and protein synthesis on 80K mRNA levels (Figure 8A). Treatment of cells with actinomycin D for 5 h had little effect on 80K mRNA, which confirms its stability, whereas 5 h incubation with cycloheximide led to a slight increase in 80K mRNA levels. Importantly, simultaneous addition of PDB with either actinomycin D or cycloheximide completely abolished the down-regulation of 80K mRNA by PDB, measured 5 h after treatment. Taken together, these results indicate that there is a requirement for both transcription and translation for the PDB-induced depletion of 80K mRNA, and suggest that the effect is mediated by the synthesis of a protein(s) which lowers 80K mRNA levels. To establish the time required for the synthesis of this protein(s), cells were exposed to PDB for 5 h, and at various times during the incubation period, RNA or protein synthesis was inhibited. Actinomycin D and the two protein synthesis inhibitors cycloheximide and anisomycin blocked the effect of phorbol ester when added up to 30 min after PDB, but had no effect when added after 2.5 h (Figure 8B). These results indicate that PDB treatment induces the synthesis of a protein(s) within 30 min of addition, which subsequently depletes 80K mRNA levels by a post-transcriptional mechanism, possibly involving a decrease in mRNA stability.

### *Effect of PDB on levels of 80K protein*

Since PDB was found to have such a dramatic effect on 80K mRNA expression, it was important to determine whether these changes are reflected at the protein level. Therefore, an antibody was raised against a C-terminal peptide of Swiss 3T3 fibroblast 80K. This antibody detects a single band corresponding to 80K in detergent extracts of Swiss 3T3 fibroblasts (Figure 9A) of intensity proportional to the amount of protein loaded (Herget and Rozengurt, unpublished observations). Quiescent cultures of these cells were treated with 800 nM PDB for various periods of time, then equal amounts of detergent solubilized proteins were analysed by Western blotting, using the specific 80K antibody. PDB treatment was found to induce a transient decrease in the abundance of the 80K protein (Figure 9). In three independent experiments, levels of this protein were found to decrease to a minimum of 25.3 ± 5.3% of control values 14−18 h after exposure to PDB. Prolonged treatment with PDB led to a slow recovery of 80K to 68.2 ± 9.5% of control values within 48 h. Therefore, the PDB-induced decrease in 80K levels reflects the effect of this phorbol ester on mRNA expression. The substantial delay observed between the effect of PDB on the expression of 80K mRNA and protein suggests that 80K is not rapidly degraded *in vivo*.

## Discussion

In this paper, we report the cDNA cloning and deduced amino acid sequence of 80K from Swiss 3T3 fibroblasts, the most prominent substrate of PKC in these cells. The identity of the clones was confirmed by direct amino acid sequencing of proteolytic peptides of the purified 80K protein (Brooks *et al.*, 1990). The complete cDNA sequence encoding 80K is 1900 bp in length, and contains a putative poly(A) tail at the 3′ end 674 bp downstream of the TAA stop codon. When compared to the deduced amino acid sequence of rat brain 80K (Erusalimsky *et al.*, 1991), there was an overall homology of 93%. In contrast, the overall homology between this protein and bovine MARCKS (Stumpo *et al.*, 1989) is only 59%, and conserved regions are largely confined to potential post-translational modification sites. The marked discrepancy between the bovine and rodent 80K proteins is intriguing, and further work is required to define their precise relationship. Two 80K mRNA species, a major band of 2.25 kb and a minor

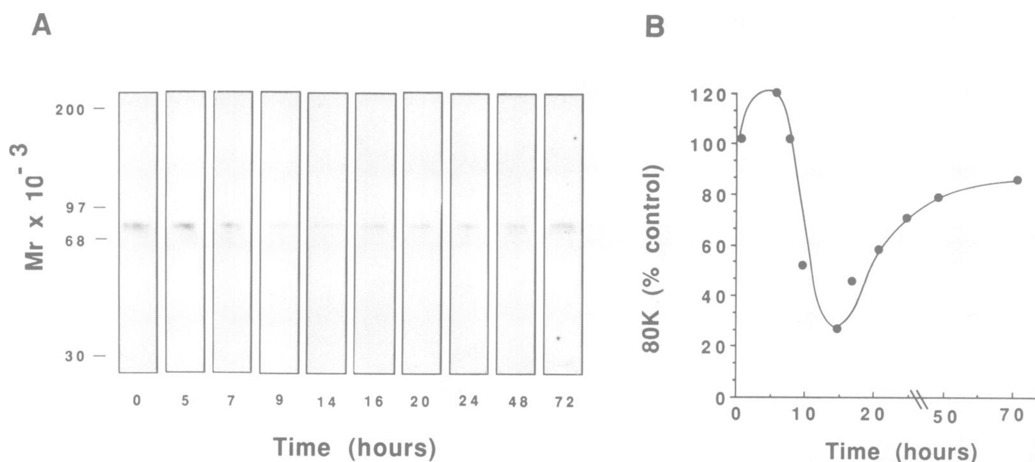


**Fig. 9.** Time course for PDB-induced depletion of 80K protein. Confluent, quiescent Swiss 3T3 fibroblasts ($1 \times 10^5$ cells) were incubated with 800 nM PDB for various periods of time. Detergent extracts were prepared from each sample and equal protein (10 $\mu$g) was analysed by Western blotting using the anti-80K antibody. A representative autoradiograph (**A**) and the corresponding results of the densitometric scans (**B**) are shown.

band of 3.9 kb, were detected by Northern blotting. Analysis of genomic DNA revealed a simple hybridization pattern consistent with a single copy of the 80K gene.

Considerable evidence demonstrates that PKC increases gene expression through a variety of nuclear transcription factors, such as AP-1 (Lee *et al.*, 1987; Angel *et al.*, 1987), NF$\kappa$B (Nelson *et al.*, 1988; Shirakawa and Mizel, 1989) and AP-2 (Imagawa *et al.*, 1987). In many cases, including c-*fos* and c-*myc*, the response is rapid and results in a marked increase in mRNA levels. These effects are often mediated by an increase in the rate of transcription, although post-transcriptional mechanisms involving alterations in mRNA stability are also important (Sherman *et al.*, 1990; Vyas *et al.*, 1990; Wisdom and Lee, 1990). In contrast, the results presented here demonstrate that activation of PKC by PDB leads to the dramatic down-regulation of 80K mRNA levels in quiescent 3T3 cells. Maximal depression occurred 5–7 h after phorbol ester addition. This effect was demonstrated with biologically active phorbol esters, including PDB, at concentrations known to bind to PKC and induce numerous biochemical responses in Swiss 3T3 fibroblasts. Furthermore, the down-regulation of 80K mRNA levels by PDB was transient, and recovery coincided with depletion of PKC. A similar transient decrease in 80K mRNA levels was also demonstrated in tertiary passage cultures of diploid mouse embryo fibroblasts. Importantly, PDB treatment of Swiss 3T3 fibroblasts also induced a transient decrease in levels of 80K within 14–18 h, thus reflecting the effects of this phorbol ester on mRNA expression. Thus, activation of PKC stimulates both the acute phosphorylation of 80K and subsequent decrease in the steady state level of both mRNA and protein. These findings may have important implications for the co-ordination of cellular signalling pathways involving PKC and $Ca^{2+}$ in view of the possible role of 80K as a calmodulin binding protein (Graff *et al.*, 1989b; McIlroy *et al.*, 1991).

The PKC-induced down-regulation of 80K mRNA could be mediated by a decrease in the rate of transcription, an increase in the rate of RNA degradation, or both. As the depletion of 80K mRNA in response to PKC activation is rapid, changes in the rate of transcription would require a constitutively higher turnover of mRNA. Since 80K mRNA is very stable, this suggests that the dramatic down-regulation of 80K mRNA seen 1–5 h after PDB treatment is mediated by a post-transcriptional mechanism which reduces the half-life of this mRNA. In support of this, PDB was found to have no effect on the rate of transcription of the 80K gene. The effect of PDB was shown to be blocked by inhibitors of both RNA and protein synthesis when added up to 30 min after PDB addition, which may implicate the product of an early response gene in this process. This protein could be a specific ribonuclease or a *trans*-acting factor which interacts with sequences carried by the 80K mRNA resulting in decreased stability.

Selective mRNA degradation is known to be an important mechanism in the control of gene expression (Cleveland, 1989), and several specific sequences or secondary structures that govern the stability of mRNAs have been reported. Among these is a 30–80 base AU-rich domain found in the 3′ untranslated region of a number of inherently unstable mRNAs, including those encoding the proto-oncogenes c-*fos* and c-*myc* and certain lymphokines and cytokines (Caput *et al.*, 1986). This domain, particularly the repetitive sequence AUUUA, may act as a recognition sequence for ribonucleases, since it also confers instability when attached to a heterologous gene (Shaw and Kamen, 1986). Other sequences which do not confer a constitutively rapid turnover but are involved in the regulation of mRNA stability have been identified. These include a conserved stem–loop structure in the extreme 3′ end of histone mRNAs (Pandey and Marzluff, 1987) and the iron response elements in the 3′ untranslated region of transferrin receptor mRNA (Muellner *et al.*, 1989; Keoller *et al.*, 1989). None of these motifs can be identified in the untranslated regions of the 80K mRNA. However, it is possible that sequences in the 80K transcripts may confer responsiveness to PKC-induced degradation. In this respect, it is of interest to note the extremely high conservation between both the 3′ and 5′ untranslated regions of the rodent 80K and bovine brain MARCKS cDNA sequences. Indeed, their 5′ untranslated regions show >90% homology, and there is >80% conservation within certain stretches of 100–200 bp in their 3′ untranslated regions. This suggests that these domains may be of regulatory importance.

To our knowledge, the results presented here provide the first report of PKC-induced down-regulation of mRNA expression mediated by a post-transcriptional mechanism. Interestingly, Schneider and co-workers reported the isolation of six growth arrest-specific genes, whose expression was shown to be down-regulated following serum stimulation of quiescent cells (Schneider *et al.*, 1988). However, the role of PKC in this response was not defined. The increasing importance of tumour suppressor genes in the development of neoplasia (Sager, 1989; Marshall, 1991) is stimulating interest in identifying genes whose expression is down-regulated by mitogenic stimulation. In this context, it will be of importance to determine whether PKC down-regulates the expression of other genes in quiescent cells and if such responses play a role in phorbol ester-induced tumour promotion.

## Materials and methods

### Cell culture

Stock cultures of Swiss 3T3 cells (Todaro and Green, 1963) were propagated as previously described (Dicker and Rozengurt, 1980). For experimental purposes, $6 \times 10^5$ cells were subcultured in 90 mm Nunc dishes with 10 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin and incubated in a humidified atmosphere of 10% $CO_2$, 90% air at 37°C. Cells were rendered quiescent by incubation under these conditions for 7 days before use. Secondary cultures of whole mouse embryo fibroblasts were subcultured in DMEM containing 10% fetal bovine serum for 3 days and then rendered quiescent by incubation in DMEM containing 0.5% fetal bovine serum for 3 days before use.

### Down-regulation of PKC

Swiss 3T3 fibroblasts have been shown to become deficient in PKC activity by prolonged incubation with PDB (Rodriguez-Pena and Rozengurt, 1984). In the present studies, confluent, quiescent cells were incubated with 800 nM PDB for 48 h in conditioned medium to down-regulate PKC, prior to the addition of specified phorbol esters. Conditioned medium is taken from cultures of Swiss 3T3 fibroblasts grown in 10% fetal bovine serum that have attained quiescence and is thus devoid of growth-promoting activity.

### Library screening and nucleotide sequencing

A Swiss 3T3 fibroblast cDNA library cloned into the *Eco*RI site of the λZAPII vector was prepared from mRNA isolated from quiescent, confluent Swiss 3T3 fibroblasts. Recombination phage ($10^6$) were screened with a probe corresponding to nucleotides 613−683 of a rat brain 80K cDNA clone (Erusalimsky *et al.*, 1991), that was labelled by filling in two oligonucleotides bearing complementary 3′ ends (underlined nucleotides) 5′-GGC-GGCGACGCGGCCGCGGCCCCCGGGGAGCAGGCCGGCGG-3′ and 5′-TCCTCGCCCTCGGCGCCCTCGGCGCCGGCGCCGCCGGC-CTGCTC-3′ with the Klenow fragment of DNA polymerase I and [α-$^{32}$P]dCTP. Filters were washed in 2 × SSC, 0.1% SDS at room temperature for 30 min followed by 0.1 × SSC, 0.1% SDS at 52°C for 1 h. Positive clones were purified, and the pBluescript phagemids excised with helper phage R408 as previously described (Erusalimsky *et al.*, 1991).

The complete cDNA sequence of 80K was obtained by custom primer-directed DNA sequencing of three overlapping clones (p803.3, p809.1 and p8017.1) using the dideoxy chain termination method (Sequenase Version 2.0 kit). Compressions and pausing problems encountered were resolved as described (Erusalimsky *et al.*, 1991). In the coding region, each strand was sequenced at least three times.

### Northern and Southern blot analyses

Confluent, quiescent cultures of fibroblasts in conditioned medium were treated with the appropriate agents as indicated in the figure legends. Total cellular RNA was prepared from these cultures by washing the monolayer twice in ice-cold phosphate-buffered saline (PBS), followed by lysis in 4 M guanidine isothiocyanate and purification by centrifugation through a caesium chloride cushion (Sambrook *et al.*, 1989). Total cellular RNA (10 μg) was electrophoresed on a 1% agarose/6% formaldehyde gel and then transferred to nylon filters. Prehybridization was performed in 5 × SSPE, 5 × Denhardt's solution, 0.1% SDS, 200 μg/ml salmon sperm DNA, 50% formamide at 42°C. The hybridization probe was a gel-purified 843 bp *Bal*I−*Eco*RI cDNA fragment spanning the 3′ end of clone p809.1 radiolabelled to a specific activity of $3 \times 10^8$ c.p.m./μg by random priming (Feinberg and Vogelstein, 1983). The $^{32}$P-labelled probe was added directly to the prehybridization solution at $10^6$ c.p.m./ml. Filters were washed in 2 × SSC, 0.1% SDS at room temperature for 15 min, followed by 0.1 × SSC, 0.1% SDS at 60°C for 30 min prior to autoradiography using Kodak X-Omat AR X-ray film at −70°C. Where appropriate, autoradiographs were scanned using an LKB Ultrosan XL laser densitometer, and the level of 80K mRNA was quantified by measuring the peak area above background.

For Southern blot analysis, genomic DNA was prepared from Swiss 3T3 fibroblasts (Sambrook *et al.*, 1989) and digested for 6 h with *Hin*dIII, *Taq*I, *Pst*I or *Eco*RI. Digested DNA (10 μg) was analysed by Southern blotting as previously described (Erusalimsky *et al.*, 1991), using the 843 bp *Bal*I−*Eco*RI cDNA fragment of p809.1 as a probe. After hybridization, the filter was washed and autoradiographed as described for Northern blotting.

### Primer extension analysis

Primer extension reactions were carried out as previously described (Herget *et al.*, 1990). A synthetic oligonucleotide probe, 5′-GGTGGAAAAGTC-GAGCACAAAAAAGGAGCCC-3′, corresponding to the antisense strand from position 20−50 bp of the 80K cDNA was used as the primer. Extension products were separated in a 6% polyacrylamide sequencing gel prior to autoradiography with Kodak X-Omat AR film.

### Transcription in isolated nuclei

Quiescent Swiss 3T3 fibroblasts were stimulated with 800 nM PDB for either 15 min or 5 h. The cells were then washed twice and harvested in 25 ml ice-cold PBS following by centrifugation at 500 *g* for 5 min. All subsequent procedures were carried out at 4°C. Nuclei were prepared by incubating the cells in 4 ml lysis buffer (0.5% NP-40, 3 mM $MgCl_2$, 10 mM NaCl, 10 mM Tris−HCl, pH 7.4) for 5 min, followed by centrifugation at 500 *g* for 5 min. The pellet was resuspended in 7.0 ml 0.32 M sucrose, 3 M $CaCl_2$, 2 mM magnesium acetate, 0.1 mM EDTA, 1 mM dithiothreitol, 10 mM Tris−HCl, pH 8.0, then centrifuged through a cushion of 7.0 ml 2 M sucrose in 5 mM magnesium acetate, 0.1 mM EDTA, 1 mM dithiothreitol, 10 mM Tris−HCl, pH 8.0, at 40 000 *g* for 40 min. Nuclei were resuspended in glycerol storage buffer (50 mM Tris−HCl, pH 8.3, 40% glycerol, 5 M $MgCl_2$, 0.1 mM EDTA) at $\sim 3 \times 10^8$/ml and frozen in 200 μl aliquots in liquid nitrogen.

The run-off transcription assay was performed using [α -$^{32}$P]UTP as described (Greenberg and Bender, 1989). As an initial purification step, the $^{32}$P-labelled RNA was subjected to a phenol:chloroform extraction procedure by the addition of 3 vol of solution D (4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.72% β-mercaptoethanol), 0.1 vol 3 M sodium acetate, pH 5.2, 3 vol phenol (pH 7.0) and 0.1 vol chloroform:isoamylalcohol (49:1). After vortexing for 1 min, the solution was incubated on ice for 30 min prior to phase separation by centrifugation at 500 *g* for 5 min. The aqueous phase was removed, and the RNA was precipitated with an equal volume of ice-cold isopropanol overnight at −20°C. After centrifugation, the RNA pellet was resuspended in 500 μl of solution D, and then reprecipitated as described above. The RNA pellet was resuspended in 50 μl of $H_2O$. Any unincorporated radiolabel was separated by centrifugation through a Sephadex G-50 spun column equilibrated in STE (0.1 M NaCl, 10 mM Tris−HCl, pH 8.0). The incorporation of [α-$^{32}$P]UTP into RNA was determined by Cerenkov counting, and equal c.p.m. ($\sim 10^6$ c.p.m./ml) were hybridized to filter-immobilized plasmids as described for Northern blotting. The target plasmids used were 2 μg pBluescript SK(−), 5 μg p809.1 (this paper), 2 μg pfosΔI, containing an 0.7 kb cDNA fragment from the coding region of v-*fos* (a gift from Dr D.Bentley), 2 μg pAG82, containing a 1.58 kb cDNA fragment from the coding region of mouse cytochrome C oxidase subunit 1 (Herget *et al.*, 1986) and 2 μg pBR18S, containing a 200 bp fragment of mouse 18S ribosomal RNA (Edwards *et al.*, 1987). Plasmids were linearized by digestion with either *Pst*I (pfosΔI) or *Eco*RI, denatured by incubation with 0.3 M NaOH for 1 h at 65°C, neutralized, and then applied to Hybond $N^+$ filters. After hybridization, the filters were washed twice with 2 × SSC, 0.1% SDS at room temperature for 10 min, and then twice with 0.1% SSC, 0.1% SDS at 55°C for 30 min prior to autoradiography with Kodak X-Omat AR X-ray film.

### Generation of antisera and immunoblotting

The synthetic peptide SPEAPPAPTAE, corresponding to residues 298−309 of the amino acid sequence of Swiss 3T3 fibroblast 80K (Figure 1), was conjugated to keyhole limpet haemocyanin and antisera were prepared as previously described (Erusalimsky *et al.*, 1991).

To determine the effect of PDB treatment of 80K levels, Swiss 3T3 fibroblasts in 30 mm dishes ($1 \times 10^5$ cells/sample) were treated with 800 nM PDB for various periods of time. After washing three times at 37°C in DMEM containing 1% bovine serum albumin, cells were incubated in 1 ml of this medium for 15 min at 37°C under 10% $CO_2$, 90% air. This washing and incubation procedure after phorbol ester treatment has been shown to remove PDB, thus causing the rapid dephosphorylation of 80K by endogenous phosphatases (Brooks and Rozengurt, unpublished observations; Rodriguez-Pena *et al.*, 1986). This eliminates any possible effects of phosphorylation on either binding to nitrocellulose or antibody recognition (Beimann and Erikson, 1990). Subsequently, cells were washed five times in ice-cold PBS and harvested in 50 μl of 2 × extraction buffer (20 mM Tris−HCl, pH 7.5, 10 mM EDTA, 2% NP-40, 1 mM PMSF, 10 μg/ml leupeptin). After incubation on ice for 10 min, insoluble material was pelleted by centrifugation at 13 000 *g* for 5 min at 4°C. Equal protein (10 μg) from the supernatant fraction was then separated by 8% SDS−PAGE. Proteins were transferred to nitrocellulose filters and stained with the Swiss 3T3 fibroblast 80K antibody (1:500 dilution) as described (Erusalimsky *et al.*, 1991), except that the filters were incubated with the antibody at 4°C overnight.

### Materials

All restriction enzymes, ultra-pure caesium chloride, guanidine isothiocyanate and the Sequenase Version 2.0 kit were from United States Biochemicals (Cleveland, OH, USA). Hybond-N, Hybond $N^+$, the Klenow fragment of DNA polymerase I, [$\alpha$-$^{35}$S]dATP (>3000 Ci/mmol) and [$\alpha$-$^{32}$P]dCTP (5000 Ci/mmol) were from Amersham International (UK). Actinomycin D, cycloheximide, leupeptin, salmon sperm DNA, phorbol-12,13-dibutyrate, phorbol 12,13,20-triacetate, phorbol and TPA were obtained from Sigma Chemical Co. Ltd (UK). Oligonucleotide probes were synthesized by Ian Goldsmith at the Imperial Cancer Research Fund. Bacterial strains of *Escherichia coli* (SURE; XLI-Blue) and helper phage R408 were from Stratagene, Cambridge, UK. T4 polynucleotide kinase was from BioLabs, New England, MA, USA, and AMV reverse transcriptase and ATP, GTP and CTP from Boehringer Mannheim (UK).

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