

# The 5' Untranslated Region of Protein Kinase C $\delta$ Directs Translation by an Internal Ribosome Entry Segment That Is Most Active in Densely Growing Cells and during Apoptosis

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**Protein kinase C $\delta$  (PKC $\delta$ ) is a member of the PKC family of phospholipid-dependent serine/threonine kinases and is involved in cell proliferation, apoptosis, and differentiation. Previous studies have suggested that different PKC isoforms might be translationally regulated. We report here that the 395-nt-long 5' untranslated region (5' UTR) of PKC $\delta$  is predicted to form very stable secondary structures with free energies ( $\Delta G$  values) of around  $-170$  kcal/mol. The 5' UTR of PKC $\delta$  can significantly repress luciferase translation in rabbit reticulocyte lysate but does not repress luciferase translation in a number of transiently transfected cell lines. By using a bicistronic luciferase reporter, we show that the 5' UTR of PKC $\delta$  contains a functional internal ribosome entry segment (IRES). The activity of the PKC $\delta$  IRES is greatest in densely growing cells and during apoptosis, when total protein synthesis and levels of full-length eukaryotic initiation factor 4G are reduced. However, the IRES activity of the 5' UTR of PKC $\delta$  is not enhanced during serum starvation, another condition shown to inhibit cap-dependent translation, suggesting that its potency is dependent on specific cellular conditions. Accumulating data suggest that PKC $\delta$  has a function as proliferating cells reach high density and in early and later events of apoptosis. Our studies suggest a mechanism whereby PKC $\delta$  synthesis can be maintained under these conditions when cap-dependent translation is inhibited.**

Protein kinase C (PKC) is a key mediator of signal transduction pathways that regulate a vast array of physiological functions, including cell proliferation, differentiation, and apoptosis (40). Members of the mammalian PKC family of phospholipid-dependent serine/threonine kinases fall into three subfamilies based on their amino acid sequences and enzymatic properties. The conventional PKCs (PKC $\alpha$ , PKC $\beta$ I, PKC $\beta$ II, and PKC $\gamma$ ) are activated by Ca<sup>2+</sup> and diacylglycerol (DAG), the novel PKCs (PKC $\epsilon$ , PKC $\delta$ , PKC $\eta$ , and PKC $\theta$ ) are DAG dependent and Ca<sup>2+</sup> independent, and the atypical PKCs (PKC $\mu$ , PKC $\zeta$ , and PKC $\iota/\lambda$ ) are Ca<sup>2+</sup> and DAG independent (7, 33). Although the heterogeneity of PKC function is postulated to be a consequence of the multiple isoforms of PKC that exist within cells, the specific roles of the various isoforms have not been clearly established.

When overexpressed, the novel PKC isoforms PKC $\epsilon$  and PKC $\delta$  have opposing effects on cell growth and proliferation. Overexpression of PKC $\epsilon$  stimulates growth and promotes transformation of rat-6 fibroblasts, NIH 3T3 fibroblasts, and rat colonic epithelial cells (2, 36, 42). However, overexpression of PKC $\delta$  inhibits growth of NIH 3T3 fibroblasts, results in cell cycle arrest of CHO cells, and is lethal to normal and neoplastic keratinocytes (24, 36, 53). PKC $\epsilon$  behaves as an oncogene because of its ability to induce tumors in nude mice (2). However, PKC $\delta$  is considered a tumor suppressor, as its overexpression reverses the transformed phenotype of D/*src* rat co-

lonic epithelial cells and depletion of PKC $\delta$  promotes *c-src*-induced transformation of rat 3Y1 fibroblasts (28, 43). Moreover, transgenic mice overexpressing PKC $\delta$  are resistant to tumor promotion by phorbol esters (45). PKC $\delta$  is also implicated in the induction and execution of programmed cell death, or apoptosis, in response to a number of signals, including ionizing radiation, tumor necrosis factor alpha, agonistic Fas antibody (10), H<sub>2</sub>O<sub>2</sub> (51), etoposide (29, 31), and phorbol esters (13, 24).

Most cellular mRNAs possess 5' untranslated regions (UTRs) of less than 100 nucleotides (nt) that are likely to allow efficient initiation of translation by cap-dependent ribosomal scanning (21). However, the 5' UTRs of many regulators of cell proliferation and apoptosis, including PKC $\epsilon$ , are long and structured and contain upstream AUG codons so that scanning ribosomes are unlikely to efficiently initiate translation (22, 39). Efficient initiation of the translation of a number of these mRNAs is achieved by a cap-independent mechanism mediated by internal ribosome entry segments (IRESs) (reviewed in reference 52). Translation mediated by IRESs is usually favored when cap-dependent initiation is inhibited. The IRESs of XIAP, p97/DAP5/NAT1, and *c-myc* are active during apoptosis (16, 17, 49). The IRES of vascular endothelial growth factor is active during hypoxia (47), and the IRES of ornithine decarboxylase is active during the G<sub>2</sub>/M phase of the cell cycle (44).

Nucleotide sequencing of PKC $\delta$  isolated from rat and mouse cDNA libraries has revealed that it has a long 5' UTR (35, 41). We show here that the 395-nt-long 5' UTR of rat PKC $\delta$  can potentially form secondary structures with free energies expected to significantly impede cap-dependent ribosome scan-

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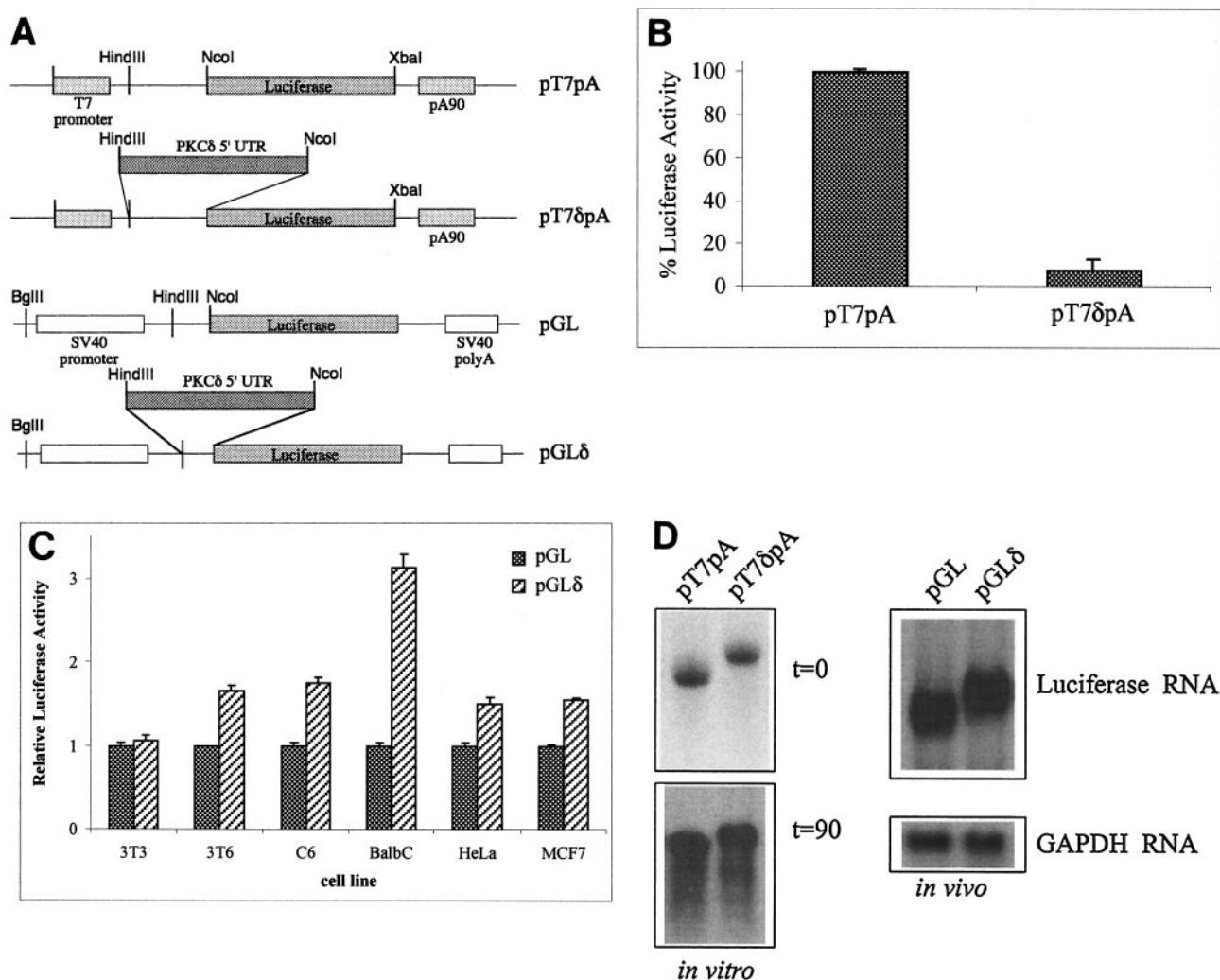


FIG. 1. The 5' UTR of PKC $\delta$  represses translation *in vitro* but not *in vivo*. (A) Schematic diagram of the T7 RNA polymerase- and pGL3-promoter-based luciferase constructs used for *in vitro* and *in vivo* analyses, respectively. The 5' UTR of PKC $\delta$  was introduced immediately upstream of the luciferase start codon in the pGL3-promoter plasmid with the *Hind*III and *Nco*I restriction sites to make pGL $\delta$ . The luciferase-coding region from pGL and the 5' UTR of PKC $\delta$  linked to luciferase from pGL $\delta$  were introduced into the pBKSpa plasmid to make pT7pA and pT7 $\delta$ pA, respectively, as described in Materials and Methods. (B) Capped and polyadenylated *in vitro*-transcribed RNAs were generated with T7 RNA polymerase from the linearized pT7pA and pT7 $\delta$ pA plasmids. Equal amounts of purified RNA were used to program rabbit reticulocyte *in vitro* translation reactions, which were subsequently assayed for luciferase activity. The luciferase activity conferred by pT7pA was normalized to 100%. Results shown are the mean and standard error of the mean from at least five independent translation reactions with different *in vitro*-transcribed RNAs. (C) Rodent and human cell lines were cotransfected with either the pGL or the pGL $\delta$  luciferase construct and pSV- $\beta$ -gal to control for transfection efficiency. For each cell line, the luciferase activity shown is relative to the  $\beta$ -galactosidase activity conferred by pSV- $\beta$ -gal. The relative luciferase/ $\beta$ -galactosidase activity conferred by pGL is normalized to a value of 1 for each cell line. Results are shown as the mean and standard error of the mean of at least five independent transfections. (D) Northern blot of pT7pA and pT7 $\delta$ pA luciferase RNAs during *in vitro* translation (top left, 0-min time point; bottom left, 90-min time point). Northern blot of RNA derived from cells transfected (*in vivo*) with the pGL and pGL $\delta$  constructs and hybridized with a  $^{32}$ P-labeled firefly luciferase (top right) or GAPDH (bottom right) riboprobe. SV40, simian virus 40.

ning and may thus be subject to translational regulation. We show that the 5' UTR of PKC $\delta$  can repress luciferase translation in rabbit reticulocyte lysate but not in transiently transfected cells. With a bicistronic reporter assay, we show that the 5' UTR of PKC $\delta$  contains an IRES and that the IRES activity of the 5' UTR of PKC $\delta$  is strongest in cells grown to a high density and during apoptosis, conditions under which cap-dependent translation is reduced. However, the IRES activity of the 5' UTR of PKC $\delta$  is not enhanced during serum starvation, another condition known to inhibit cap-dependent translation.

#### MATERIALS AND METHODS

**DNA constructs.** The PKC $\delta$  cDNA clone (41) was derived from rat brain and was a gift from Y. Ono (Kobe University, Kobe, Japan). The luciferase reporter plasmids pGL3-promoter (called pGL here) and pGL3-control (called pGLenh here), the pSV- $\beta$ -galactosidase plasmid, and cloning plasmid pGEM7Zf(-) were purchased from Promega. Restriction and modifying enzymes were purchased from New England Biolabs or Promega and used in accordance with the manufacturer's instructions. Plasmid DNA was prepared for transfection with a plasmid midi prep kit (QIAGEN).

Plasmid constructs for transient transfection were generated as follows. A 3-kb *Eco*RI fragment comprising the cDNA of PKC $\delta$  was subcloned into pGEM7.

The 5' UTR was cloned by PCR with an SP6 primer (5' end of the 5' UTR) and a PKC $\delta$ -specific primer from the start codon, introducing a *NcoI* site (CCATGG) as the start codon. The PCR product was cleaved with *HindIII/NcoI* to release a 390-bp fragment that was subcloned into the pGL3-promoter plasmid and the pGL3-control plasmid to make pGL $\delta$  and pGLenh- $\delta$ , respectively. The following plasmids were generously provided by A. Willis (Leicester University, Leicester, United Kingdom) and M. Stoneyley (Leeds University, Leeds, United Kingdom): pGL3R (called pR-F here), pGL3RutrH (48), pGL3RHRV (called pR-HRV-F here) (49), and pHpL (called pH here) (50). The 5' UTR of PKC $\delta$  was introduced between the *Renilla* and firefly luciferase coding regions with the *EcoRI* and *NcoI* restriction sites to make pR- $\delta$ -F. Bicistronic plasmid pGL3RutrH, containing a stable hairpin 5' of *Renilla* luciferase, was used to make pHR- $\delta$ -F by exchanging the 5' UTR of *c-myc* with the 5' UTR of PKC $\delta$  with the *SpeI* and *NcoI* restriction sites. The pHpL monocistronic luciferase plasmid, containing a stable hairpin 5' of the luciferase start, was used to make pH- $\delta$  with the *EcoRI* and *NcoI* restriction sites.

For the plasmids used for in vitro transcription/translation, *HindIII*-to-*XbaI* fragments from the pGL3-promoter plasmid (containing the entire luciferase coding region) and the same restriction fragment from pGL $\delta$  (containing the 5' UTR of PKC $\delta$  linked to the luciferase open reading frame) were treated with the Klenow fragment of DNA polymerase to generate blunt ends and subcloned into the *SmaI* site of the T7 promoter pA90 vector pBKSpA (a gift from James Malter) to make pT7pA and pT7 $\delta$ pA, respectively. The integrity of all clones was confirmed by DNA sequencing.

**In vitro transcription and translation.** In vitro-transcribed, capped RNA was made with T7 RNA polymerase (Gibco BRL) in a 20- $\mu$ l reaction mixture comprising 1  $\mu$ g of *HindIII*-linearized pT7pA or pT7 $\delta$ pA plasmid DNA; 0.5 mM (each) CTP, UTP, and ATP; and 0.05 mM GTP with 0.5 mM 7mG cap analogue (Promega) in 1 $\times$  T7 transcription buffer (40 mM Tris/HCl [pH 8.0], 8 mM MgCl<sub>2</sub>, 2 mM spermidine, 25 mM NaCl), 5 mM dithiothreitol, and 30 U of RNasin (Promega) at 37°C for 2 h. The reaction mixture was treated with DNase I and purified through G-50 Sephadex columns (Pharmacia) to remove unincorporated nucleotides and free 7mG cap. The RNA was quantified spectrophotometrically, and its quality was verified on nondenaturing agarose gels. Equal amounts of RNA (typical final concentration, 20 ng/ $\mu$ l) were used in a 25- $\mu$ l in vitro translation reaction mixture comprising 70% rabbit reticulocyte lysate (Promega), 0.02 mM each amino acid, and 30 U of RNasin. The in vitro translation was allowed to proceed at 28°C for 90 min, and the reaction mixture was finally treated with 1  $\mu$ l of RNase A at 10 mg/ml for 10 min at 28°C. One microliter of each reaction mixture was used in a luciferase assay (with 50  $\mu$ l of luciferase assay reagent [described below]). A 1- $\mu$ l sample was taken at the start and end of the translation reaction, and the RNA was extracted for Northern blot analysis. The translation reactions were done at least five times with different batches of in vitro-transcribed RNA and rabbit reticulocyte lysate.

**Cell culture and transient transfection.** The 3T3 Swiss albino (mouse fibroblast), 3T6 Swiss albino (mouse fibroblast), BALB/3T12-3 (mouse tumorigenic fibroblast), C6 (rat glioma), HeLa (human cervical carcinoma), and MCF7 (human breast carcinoma) cell lines were purchased from ECACC (Salisbury, United Kingdom) and maintained at 37°C in 5% CO<sub>2</sub> in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Life Technologies) containing penicillin/streptomycin and supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Life Technologies). For a typical transient transfection, cells were grown to 50 to 70% density in six-well plates and then cotransfected with 1  $\mu$ g of pSV- $\beta$ -galactosidase plasmid and 1  $\mu$ g of the luciferase reporter plasmid with 10  $\mu$ l (30  $\mu$ g) of Superfect reagent (QIAGEN) in accordance with the manufacturer's instructions. Low, medium, and high cell densities were achieved by seeding cells at  $4 \times 10^3$ /cm<sup>2</sup> (approximately 10% density),  $2 \times 10^4$ /cm<sup>2</sup> (approximately 50% density), or  $4 \times 10^4$ /cm<sup>2</sup> (approximately 100% density), respectively. Cells were transfected at these densities and then harvested after 24 h. For serum starvation experiments, cells growing at 50 to 70% density in 10% FBS-containing DMEM were transfected, and then 6 h later, the cells were washed twice with serum-free DMEM and grown for a further 24 h in serum-free DMEM before being harvested. To induce apoptosis, 0.5  $\mu$ g of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was added per ml of medium 24 h after transfection and the cells were harvested 6 h later. For the RNA analysis, a 75-cm<sup>2</sup> flask of cells was transfected for each construct with 10  $\mu$ g of plasmid DNA and 15  $\mu$ l of FuGene 6 (Roche) in accordance with the manufacturer's instructions.

**Enzyme assays.** For the monocistronic luciferase assays, cells were harvested by being scraped into 120  $\mu$ l of reporter lysis buffer and assayed with the Luciferase Assay System (Promega) in accordance with the manufacturer's instructions. For the bicistronic luciferase assays, cells were harvested with 120  $\mu$ l of passive lysis buffer and assayed with the Dual Luciferase Reporter Assay System (Promega) in accordance with the manufacturer's instructions. The peak light

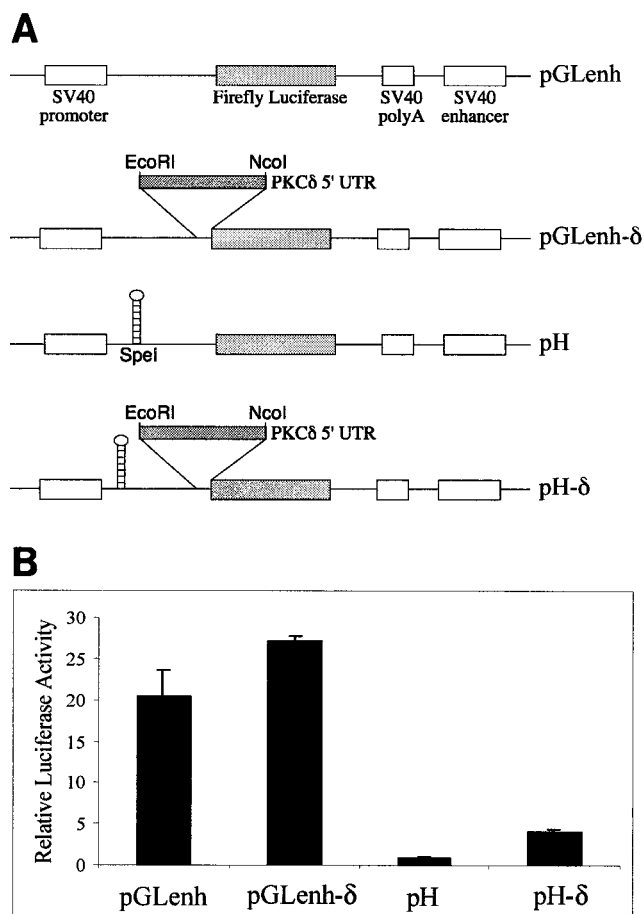


FIG. 2. The 5' UTR of PKC $\delta$  enhances translation when cap-dependent scanning is inhibited in a monocistronic construct. (A) Schematic diagram of the constructs used. The 5' UTR of PKC $\delta$  was introduced immediately upstream of the luciferase open reading frame in the pGL3-control plasmid (called pGLenh) to create pGLenh- $\delta$ . The stable hairpin structure with a free energy ( $\Delta G$ ) of  $-55$  kcal/mol was introduced 5' of the firefly luciferase start in the pGLenh plasmid to create pH as previously described (50). The 5' UTR of PKC $\delta$  was introduced between the hairpin and the luciferase start to create pH- $\delta$  as shown. (B) Luciferase activities conferred by the monocistronic hairpin constructs in transiently transfected HeLa cells relative to the  $\beta$ -galactosidase activity conferred by pSV- $\beta$ -gal to control for transfection efficiency. The relative luciferase or  $\beta$ -galactosidase activity conferred by the pH construct is normalized to a value of 1. The results shown are the mean and standard error of the mean of at least five independent transfections. SV40, simian virus 40.

emission for 10 s at 25°C due to luciferase activity was measured as luciferase relative light units (LRLUs) with a Berthold luminometer.  $\beta$ -Galactosidase activity was measured with the substrate *o*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG).  $\beta$ -Galactosidase activity absorbances were measured at 420 nm. For each transfection, the luciferase activity is expressed relative to the  $\beta$ -galactosidase activity to control for transfection efficiency. The mean values of relative luciferase activity shown in Results were derived from the analysis of at least five independent cotransfections of each luciferase plasmid with pSV- $\beta$ -galactosidase with at least two different preparations of plasmid DNA.

**RNA analysis.** RNA secondary structures were predicted with mFOLD (32), which is available at <http://bioinfo.math.rpi.edu/~mfold/rna/form1.cgi>. Total RNA was extracted with the Trizol reagent (Gibco BRL, Life Technologies) and treated with RQ1 DNase (Promega). Polyadenylated RNA was purified with oligo(dT) cellulose with the PolyA-spin mRNA isolation kit (New England Biolabs). RNA from transiently transfected cells [total: 20  $\mu$ g of luciferase RNA, 10  $\mu$ g of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA, or 1  $\mu$ g of

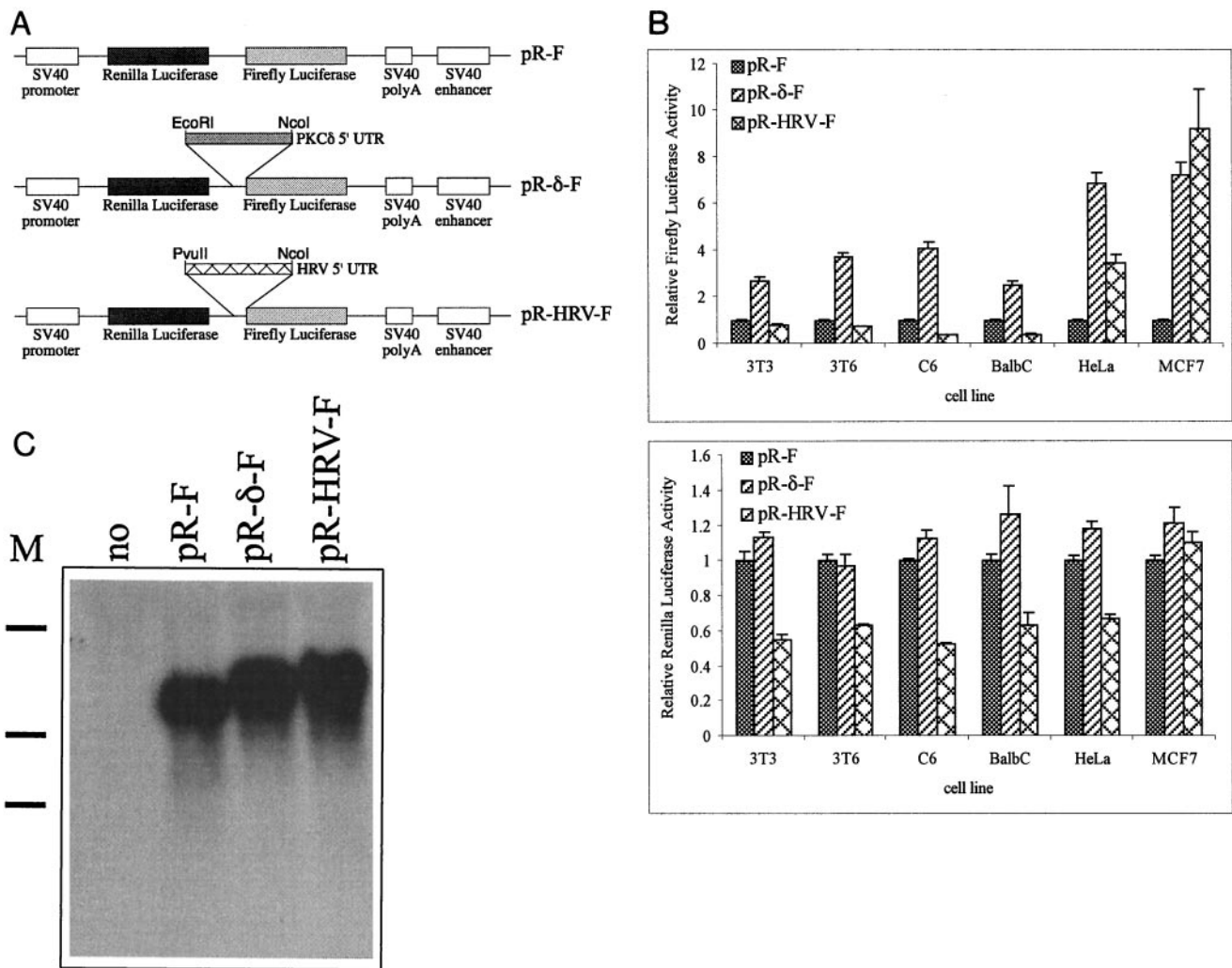


FIG. 3. The 5' UTR of *PKC $\delta$*  acts as an IRES in a bicistronic construct. (A) Schematic diagram of bicistronic *Renilla* and firefly luciferase constructs used to test for IRES activity in transiently transfected cells. The 5' UTR of *PKC $\delta$*  was inserted between the coding regions of the *Renilla* and firefly luciferases in the pR-F vector to make pR- $\delta$ -F as detailed in Materials and Methods. The pR-HRV-F construct containing the HRV IRES was used as a positive control (49). (B) Firefly and *Renilla* luciferase activities conferred by the bicistronic constructs in transiently transfected rodent and human cell lines relative to the  $\beta$ -galactosidase activity conferred by pSV- $\beta$ -gal. For each cell line, the relative firefly and *Renilla* luciferase activities conferred by pR-F are normalized to a value of 1. The results shown are the mean and standard error of the mean from at least five independent transfections. (C) Northern blot analysis of 1  $\mu$ g of poly(A) RNA isolated from HeLa cells transiently transfected with no DNA or the pR-F, pR- $\delta$ -F, or pR-HRV-F construct and hybridized with a firefly luciferase-specific  $^{32}$ P-labeled riboprobe. The approximate positions (from top of gel to bottom) of 5-, 3-, and 2-kb RNA markers (M) are indicated on the left.

poly(A) RNA] was denatured with formamide/formaldehyde and subjected to electrophoresis on a 1% agarose–6% formaldehyde–morpholinepropanesulfonic acid (MOPS) gel. The RNA was transferred onto Hybond N<sup>+</sup> membranes (Amersham). The membranes were hybridized with [ $\alpha$ - $^{32}$ P]UTP (Amersham Radiochemicals)-labeled single-stranded RNA antisense probes derived from the entire coding regions of firefly luciferase or GAPDH (12) in ULTRAhybe hybridization buffer (Ambion). The luciferase and GAPDH RNA signals were quantified by phosphorimaging with a Molecular Dynamics phosphorimager with ImageQuant (version 5.1) software.

*PKC $\delta$*  and GAPDH RNA levels were assessed in cells growing at different densities with semiquantitative reverse transcription (RT)-PCR. One microgram of total RNA was reverse transcribed with 0.5  $\mu$ g of 5'-pd(T)<sub>12-18</sub>-3' (Pharmacia) with SuperScript II reverse transcriptase (Gibco BRL, Life Technologies) in a 25- $\mu$ l reaction volume in accordance with the manufacturer's instructions. The PCR was performed in a 50- $\mu$ l volume with 2  $\mu$ l of the RT reaction mixture with *Taq* polymerase (Promega) in accordance with the manufacturer's instructions. The number of amplification cycles chosen for analysis of *PKC $\delta$*  and GAPDH

RNA levels by semiquantitative PCR was determined for each primer pair to be within the exponential range for cells growing at different densities. *PKC $\delta$*  was amplified with primers specific to the C-terminal variable region (sequences: 5' GAC ATC ATG GAG AAG CTA TTC and 5' TCG ATG AGG TTC TTG TCA CTG) with 20 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and GAPDH was amplified with specific primers (sequences: 5' AGA CAG CCG CAT CTT CTT GTG C and 5' CTC CTG GAA GAT GGT GAT GG) with 15 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C, for 1 min. Ten microliters of the PCR mixture was electrophoresed on 2% agarose gels and stained with ethidium bromide. The intensity of ethidium bromide staining was quantified with the AlphaImager 1200 documentation and analysis system (Alpha Innotech Corporation). The specificity of the PCR products was confirmed by Southern blotting with oligonucleotide probes designed to hybridize to internal sequences of the predicted PCR products (data not shown).

**Metabolic labeling of cells.** Cells were grown in serum-free methionine/cysteine-free DMEM for 30 min prior to the addition of 60  $\mu$ Ci of [ $^{35}$ S]Met/Cys (Promix; Amersham) per ml and incubation for 1 h. Cells were then washed twice

with phosphate-buffered saline and harvested in lysis buffer A (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 10  $\mu$ g of aprotinin per ml, 10  $\mu$ g of leupeptin per ml, and 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF). Total protein concentrations were estimated with the bicinchoninic acid assay (Pierce), and equal amounts of protein were added to Laemmli loading buffer and heated to 100°C for 5 min before being subjected to denaturing polyacrylamide gel electrophoresis with 10% polyacrylamide gels. Proteins were transferred to Hybond-C (Amersham) membranes. The membranes were stained with Ponceau (Sigma) to visualize the protein, individual lanes were excised, and the amount of incorporated  $^{35}$ S in each lane was quantified by scintillation counting.

**Immunoblotting.** Total protein was extracted from cells by scraping them into lysis buffer A containing 10  $\mu$ g of aprotinin per ml, 10  $\mu$ g of leupeptin per ml, and 2 mM AEBSF for eukaryotic initiation factor 4G (eIF4G) or by scraping them into lysis buffer B (0.5% NP-40, 50 mM Tris, 0.5 mM EDTA, 2 mM EGTA, pH 7.5) containing 10  $\mu$ g of aprotinin per ml, 10  $\mu$ g of leupeptin per ml, and 2 mM AEBSF for PKC $\delta$ . Samples containing 30  $\mu$ g of protein were subjected to denaturing polyacrylamide gel electrophoresis and transferred to Hybond-C as described above. The membranes were incubated with polyclonal rabbit antiserum against PKC $\delta$  (used at 1:5,000; a gift from N. Groom, Oxford Brookes University), with polyclonal rabbit antiserum against the C terminus (amino acids 920 to 1396) of eIF4G1 (used at 1:2,000; a gift from S. Morley, Sussex University), or with polyclonal rabbit antiserum against poly(ADP-ribose) polymerase (PARP), which detects both the 116-kDa full-length PARP molecule and the 89-kDa caspase cleavage product (used at 1:1,000; purchased from Cell Signaling Technology). The membranes were then incubated with an anti-rabbit peroxidase-conjugated secondary antibody (used at 1:5,000; Sigma). Immunoreactive bands were detected by chemiluminescence assay.

## RESULTS

**The 5' UTR of PKC $\delta$  is predicted to form highly stable secondary structures and is resistant to cap-dependent translation in vitro but not in vivo.** The 5' UTR of PKC $\delta$  isolated from a rat brain cDNA library is 395 nt long and has a G+C content of 68%. With the computer algorithm of Zuker (32), which estimates the minimum free energy of a folded RNA molecule, the 5' UTR of PKC $\delta$  was predicted to form highly stable secondary structures with free energies ( $\Delta G$  values) exceeding  $-170$  kcal/mol (data not shown). As an RNA stem-loop structure with a free energy of  $-50$  kcal/mol is inhibitory to cap-dependent ribosomal scanning (20), it was expected that the 5' UTR of PKC $\delta$  would inhibit cap-dependent translation initiation. To test this, the 5' UTR of PKC $\delta$  was introduced immediately upstream of the luciferase start in either T7 RNA polymerase promoter-based plasmid pT7pA or simian virus 40 promoter-based plasmid pGL to create pT7 $\delta$ pA and pGL $\delta$ , respectively, as shown in Fig. 1A. Capped and polyadenylated luciferase RNA generated in vitro from pT7 $\delta$ pA with T7 RNA polymerase was poorly translated in rabbit reticulocyte lysate compared to luciferase RNA generated from the control, pT7pA (Fig. 1B). In transiently transfected Swiss 3T3 cells, the levels of luciferase conferred by the pGL and pGL $\delta$  constructs were similar (Fig. 1C). However, in transiently transfected Swiss 3T6, C6, BALB/c, HeLa, and MCF7 cells, the pGL $\delta$  construct conferred significantly more luciferase activity than did pGL (*t* test, *P* < 0.01) (Fig. 1C). Northern blot analysis revealed that comparable amounts of luciferase-specific RNA of the expected size could be recovered after 90 min from the in vitro translation reactions programmed with in vitro-transcribed pT7pA and pT7 $\delta$ pA (Fig. 1D). In transiently transfected cells, the pGL and pGL $\delta$  constructs produced similar levels of luciferase mRNA of the expected size. These results suggest that, in contrast to the pT7pA and pGL constructs, the pT7 $\delta$ pA and pGL $\delta$  constructs do not affect steady-state levels

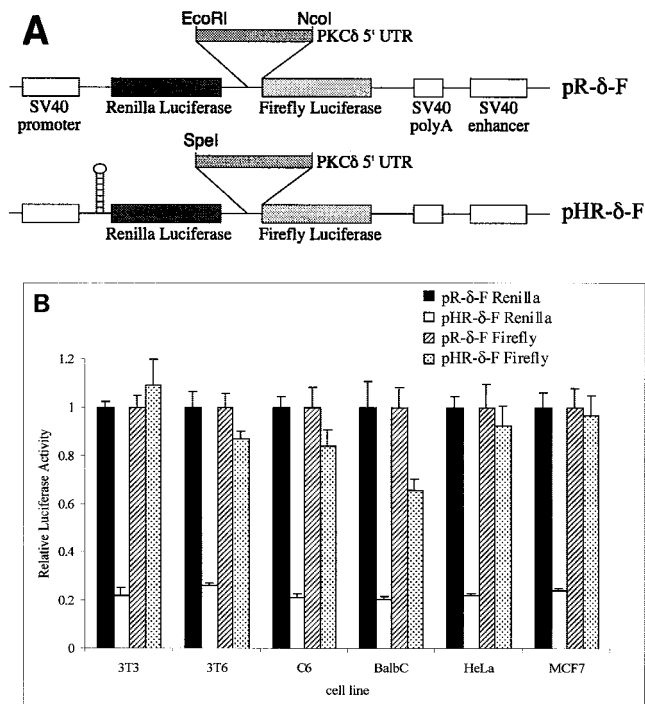


FIG. 4. The 5' UTR of PKC $\delta$  does not promote ribosomal readthrough in the bicistronic construct. (A) Schematic diagram of the bicistronic constructs used for transient transfections. The stable hairpin structure with a free-energy of  $-55$  kcal/mol introduced 5' of the *Renilla* luciferase start (48) was used to create pHR- $\delta$ -F. (B) *Renilla* and firefly luciferase activities conferred by the bicistronic pR- $\delta$ -F and pHR- $\delta$ -F constructs in transiently transfected rodent and human cell lines relative to the  $\beta$ -galactosidase activity conferred by pSV- $\beta$ -gal. For each cell line, the relative *Renilla* and firefly luciferase activities conferred by the pR- $\delta$ -F construct were normalized to a value of 1. The results shown are the mean and standard error of the mean from at least five independent transfections.

of luciferase mRNA, nor are they likely to contain splicing sequences. Taken together, these data suggest that the 5' UTR of PKC $\delta$  can repress luciferase translation in rabbit reticulocyte lysate but enhance translation of luciferase in several cell lines.

**The 5' UTR of PKC $\delta$  enhances translation when cap-dependent ribosomal scanning is inhibited.** HeLa cells were transiently transfected with a construct carrying a stable hairpin structure designed to block cap-dependent translation (Fig. 2A). Luciferase activity conferred by the pH construct containing the stable hairpin structure was 5% of that conferred by the control, pGLenh (Fig. 2B). The pH- $\delta$  construct carrying the 5' UTR of PKC $\delta$  between the stable hairpin and the translation start site conferred fourfold more luciferase activity than did pH, suggesting that the 5' UTR of PKC $\delta$  can promote translation initiation when cap-dependent scanning is inhibited (Fig. 2B). However, luciferase activity conferred by the pH- $\delta$  construct was around fivefold less than that conferred by the control, pGLenh, and more than sixfold less than that conferred by pGLenh- $\delta$ . This suggests that a component of translation initiation by the 5' UTR of PKC $\delta$  must be cap dependent and has been inhibited by the introduced cap-proximal hairpin. Alternatively, the cap-proximal hairpin may have in-

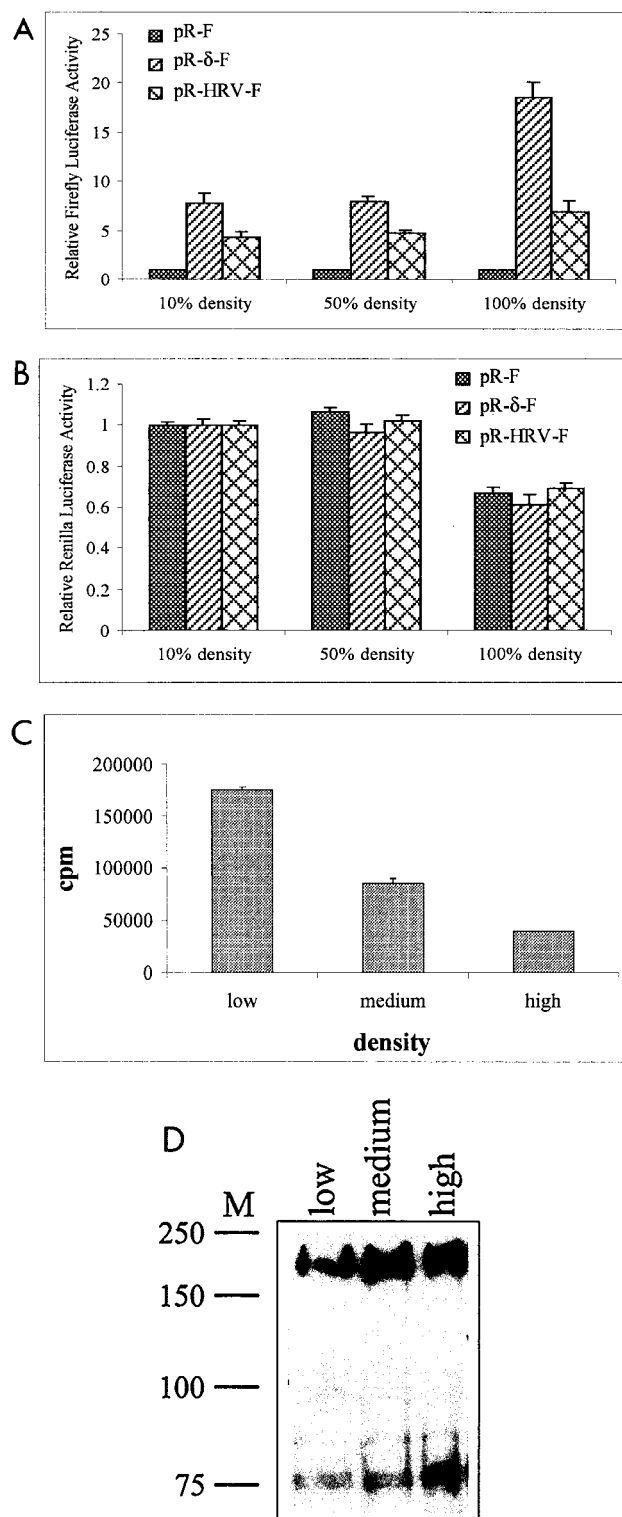


FIG. 5. The IRES activity of the 5' UTR of PKC $\delta$  is greatest in densely growing cells. (A) Firefly luciferase activities conferred by the bicistronic pR-F, pR- $\delta$ -F, and pR-HRV-F constructs in HeLa cells transiently transfected at approximately 10, 50, and 100% cell density relative to the  $\beta$ -galactosidase activity conferred by pSV- $\beta$ -gal. Relative firefly luciferase activity conferred by pR-F is normalized to a value of 1 for each cell density. (B) Relative *Renilla* luciferase activity conferred by the bicistronic constructs, normalized to a value of 1 for each construct at 10% cell density. The results shown are the mean and

standard error of the mean from at least five independent transfections. (C) Global protein synthesis was determined by labeling HeLa cells growing at a low (10 to 20%), medium (50 to 70%), or high (90 to 100%) density with [ $^{35}$ S]methionine as described in Materials and Methods. (D) Total protein extracted from cultures parallel to those in panel C were electrophoresed through SDS-10% acrylamide gels, and the proteins were transferred to nitrocellulose and probed with antisera raised against eIF4G. The approximate locations of protein molecular size markers (M) in kilodaltons are indicated at the left.

interfered with the function of the 5' UTR of PKC $\delta$  in promoting cap-independent initiation.

**The 5' UTR of PKC $\delta$  functions as an IRES in a bicistronic construct.** The 5' UTR of PKC $\delta$  was introduced between the *Renilla* and firefly luciferase coding regions in a bicistronic reporter plasmid to test if it could function as an IRES in vivo (Fig. 3A). The human rhinovirus (HRV) IRES was included in the transfections as a positive control (1, 49). In all of the cell lines tested, the presence of the 5' UTR of PKC $\delta$  in the intergenic region resulted in downstream (firefly) luciferase activity two- to sevenfold greater than that of the control, pR-F (Fig. 3B). There were no significant differences in the activity of the upstream (*Renilla*) luciferase conferred by the pR-F and pR- $\delta$ -F constructs in any of the transiently transfected cells (Fig. 3B). The 5' UTR of PKC $\delta$  was at least as efficient as the HRV IRES in initiating translation of the downstream luciferase in cells of human origin and was at least twofold more efficient in cells of rodent origin. The lack of activity of the HRV IRES in nonhuman cells is consistent with previous studies (1). Northern blot analysis of mRNA isolated from HeLa cells transiently transfected with the control, PKC $\delta$ , and HRV bicistronic constructs revealed an intact single species of luciferase-specific mRNA migrating at the expected size (Fig. 3C). This supports the notion that the 5' UTRs of PKC $\delta$  and HRV neither alter the steady-state levels of the bicistronic RNA nor result in cleavage of the bicistronic RNA into a monocistronic firefly luciferase.

**The 5' UTR of PKC $\delta$  does not promote ribosomal readthrough in the bicistronic construct.** The 5' UTR of PKC $\delta$  may function in the bicistronic construct by stimulating readthrough of ribosomes through the intergenic region. If this were the case, it would be expected that, in the presence of a stable hairpin that inhibits translation from the upstream cistron, the level of luciferase activity from the downstream cistron would be proportionally decreased in constructs carrying the 5' UTR of PKC $\delta$  as the intergenic spacer. We found that in the control bicistronic construct, which lacks an intergenic spacer, a stable hairpin structure inserted 5' of the *Renilla* coding region reduced translation of both luciferase cistrons to around 20% of the levels conferred by the control lacking the hairpin (reference 48 and data not shown). We compared the *Renilla* and firefly luciferase activities in the bicistronic construct carrying the 5' UTR of PKC $\delta$  as the intergenic spacer in the absence and presence of a stable hairpin 5' of the upstream cistron (Fig. 4A). Although *Renilla* luciferase activity was significantly reduced in all of the cell lines tested, there was no significant reduction in firefly luciferase activity (Fig. 4B). This confirms that the 5' UTR of PKC $\delta$  does not function by enhancing readthrough of the bicistronic construct and supports the notion that the 5' UTR of PKC $\delta$  contains a bona fide IRES.

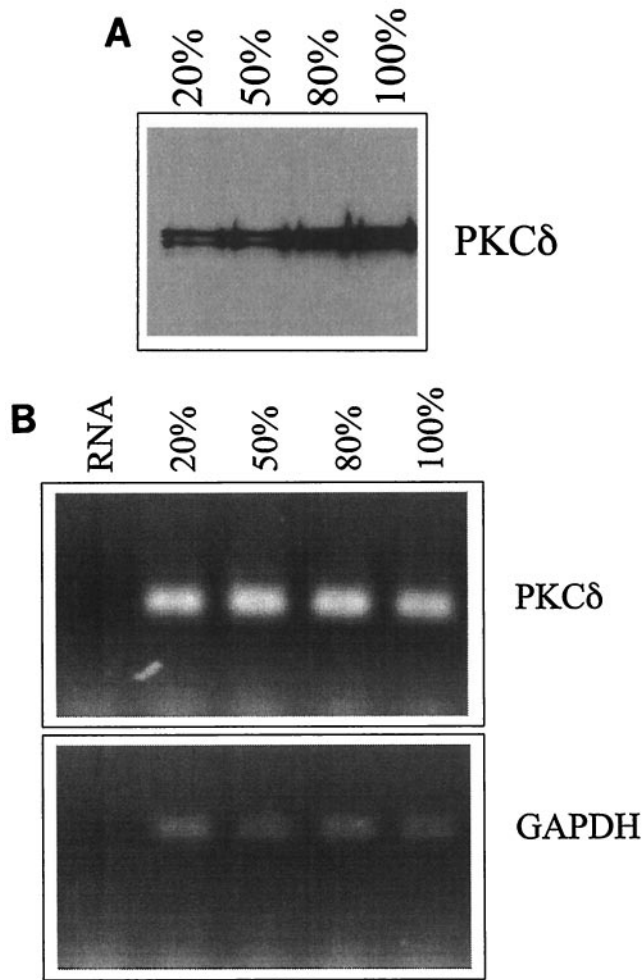


FIG. 6. Levels of endogenous PKC $\delta$  protein, but not its mRNA, increase in densely growing cells. (A) Equal amounts of total protein extracted from HeLa cells growing at approximately 20, 50, 80, and 100% density were electrophoresed through SDS-10% acrylamide gels and then transferred to nitrocellulose and probed with antiserum against PKC $\delta$ . (B) PKC $\delta$  RNA levels were determined with semiquantitative RT-PCR with total RNA isolated from HeLa cells growing at approximately 20, 50, 80, and 100% density (upper band, PCR product; lower band, primer). The same reverse-transcribed RNA samples were amplified with primers specific for GAPDH. A negative control of amplified RNA is shown in the left lane of each gel.

**The IRES activity of the 5' UTR of PKC $\delta$  is greatest in cells growing at a high density, when cap-dependent translation is reduced.** Previous studies have indicated that IRES activity of a 5' UTR can promote translation initiation during times of cellular stress and other occasions when cap-dependent translation is inhibited. Cells grown to a high density, or confluency, enter G<sub>0</sub> and growth arrest, which is marked by changes in the expression of many cellular factors (46, 54). PKC $\delta$  protein levels are maximal when RPTE cells, C6 rat glioma cells, and 3T6 fibroblasts are grown to a high density rather than a subconfluent cell density (9, 27, 38). We therefore speculated that the IRES activity of the 5' UTR of PKC $\delta$  might be required to translate the mRNA when cells are grown to a high density. To test this, HeLa cells were transiently transfected with the con-

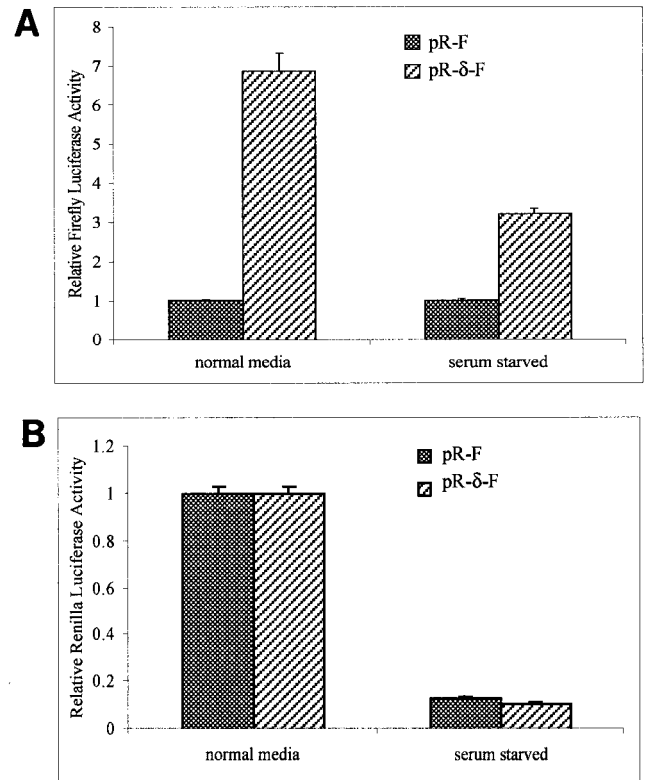


FIG. 7. The IRES activity of the 5' UTR of PKC $\delta$  is not enhanced in serum-starved cells. (A) HeLa cells were grown in 10% FBS-containing DMEM to approximately 50% density and transiently transfected with the bicistronic pR-F or pR- $\delta$ -F construct and pSV- $\beta$ -gal to control for transfection efficiency. After 6 h, the cells were washed twice with serum-free DMEM and incubated in serum-free DMEM for a further 24 h before being harvested and assayed for firefly luciferase and  $\beta$ -galactosidase activities. Relative firefly luciferase activity conferred by pR-F is normalized to a value of 1 for each condition. (B) Relative *Renilla* luciferase activities from the transfected cells described above were determined and normalized to a value of 1 for each construct in cells growing in normal medium. The results shown are the mean and standard error of the mean from at least five independent transfections.

trol, PKC $\delta$ , and HRV bicistronic constructs at low, medium, and high cell densities. At low and medium cell densities, the level of firefly luciferase activity conferred by pR- $\delta$ -F was around eightfold greater than that of the control, pR-F (Fig. 5A). However, when cells were transfected at a high density, the level of firefly luciferase activity conferred by pR- $\delta$ -F was 18-fold greater than that conferred by the control. Elevated firefly luciferase activity conferred by pR- $\delta$ -F compared to the control was also observed when the cells were transfected at a medium density and then harvested after growth to a high density (data not shown). There was a modest, but not statistically significant, improvement in the IRES activity of the HRV construct in cells growing at a high density compared to that of cells growing at a medium density. These data therefore suggest that the IRES activity of the 5' UTR of PKC $\delta$  is specifically elevated in cells growing at a high density. It is likely that cap-dependent translation is reduced in cells growing at a high density, as the relative amount of the upstream

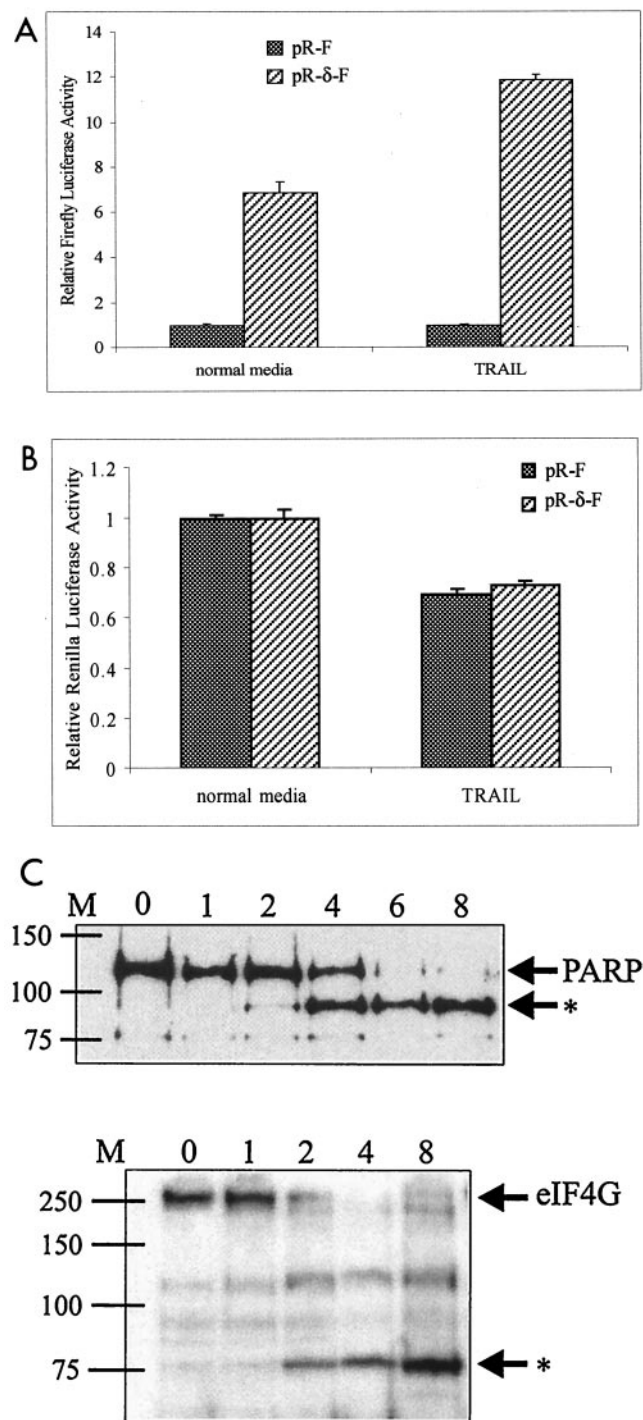


FIG. 8. The IRES activity of the 5' UTR of PKC $\delta$  is enhanced in cells treated with TRAIL to induce apoptosis. (A) HeLa cells were transiently transfected with the bicistronic pR-F or pR- $\delta$ -F construct and pSV- $\beta$ -gal to control for transfection efficiency. After 24 h, 0.5  $\mu$ g of TRAIL per ml was added to the medium and the cells were harvested 6 h later. Relative firefly luciferase activities are shown, with the levels conferred by pR-F normalized to a value of 1. (B) Relative *Renilla* luciferase activities from the transiently transfected cells above were determined and normalized to a value of 1 for each construct in cells in normal medium. (C) Equal amounts of total protein extracted from HeLa cells growing in the presence of 0.5  $\mu$ g of TRAIL per ml for 0, 1, 2, 4, 6, and 8 h were electrophoresed through SDS-10% acrylamide gels and then transferred to nitrocellulose and probed with

(*Renilla*) luciferase was significantly reduced under these growth conditions compared to that in low- and medium-density cells (Fig. 5B). However, it is also possible that the half-life of *Renilla* luciferase is shorter in cells growing at a high density and that cap-dependent translation is not reduced in densely growing cells. To test whether cap-dependent translation is reduced in densely growing cells, HeLa cells growing at different densities were labeled with [ $^{35}$ S]methionine for 1 h prior to being harvested (Fig. 5C). Analysis of the cell lysates revealed reduced incorporation of [ $^{35}$ S]methionine at a high cell density, supporting the idea that global cell protein synthesis is reduced. A significant reduction in global protein synthesis was also observed in 3T6 cells grown to a high density (data not shown). Proteolytic cleavage of eIF4G has previously been observed under conditions in which global translation is reduced (5, 30). As HeLa cells grow to a high density, immunoblotting for eIF4G revealed a predicted 76-kDa cleavage product (Fig. 5D). Taken together, these results suggest that when cells are growing at a high density, global translation is reduced and the IRES activity of the 5' UTR of PKC $\delta$  is significantly enhanced.

**Endogenous PKC $\delta$  protein levels are elevated as cells reach a high density, while its mRNA levels do not change.** To determine the status of endogenous PKC $\delta$  in HeLa cells growing at different densities, we performed immunoblotting and semi-quantitative RT-PCR. Immunoblotting revealed that PKC $\delta$  protein levels peak in cells growing at a maximal density (Fig. 6A), while there was no change in the PKC $\delta$  mRNA levels (Fig. 6B). This suggests that as HeLa cells grow to a high density, endogenous PKC $\delta$  protein levels are modulated post-transcriptionally.

**The IRES activity of the 5' UTR of PKC $\delta$  is not enhanced by serum starvation but is enhanced during apoptosis.** Previous studies have demonstrated that serum starvation and apoptosis reduce global translation (5, 49). We therefore examined whether the IRES activity of the 5' UTR of PKC $\delta$  is improved under these conditions of cellular stress. Cells were transfected with the bicistronic constructs and then subjected to serum starvation or apoptosis induced by TRAIL. During serum starvation, there was a significant reduction in both firefly (cap-independent) and *Renilla* (cap-dependent) luciferase activities (Fig. 7A and B). This suggests that the IRES activity of the 5' UTR of PKC $\delta$  is diminished during serum starvation.

The IRES activity of the 5' UTR of *c-myc* is maintained during apoptosis induced by TRAIL (49). In HeLa cells transfected with pR- $\delta$ -F, we observed a significant improvement in relative firefly luciferase activity, compared to that of the pR-F control, 6 h after 0.5  $\mu$ g of TRAIL per ml was added to the culture medium (Fig. 8A). Cap-dependent initiation was modestly but significantly reduced, as shown by analysis of the *Renilla* luciferase activity (Fig. 8B). Induction of apoptosis by 0.5  $\mu$ g of TRAIL per ml is clearly evident after 4 h by the appearance of the 89-kDa caspase-specific cleavage product of PARP (Fig. 8C). Further evidence that TRAIL reduces cap-

antisera against PARP or eIF4G. The positions of full-length PARP and eIF4G are indicated, and the position of specific cleavage products is shown by the asterisk. The approximate locations of protein molecular size markers (M) in kilodaltons are indicated at the left.



dependent translation is shown by a time-dependent reduction of the full-length eIF4G-specific signal and the appearance of the 76-kDa cleavage product 2 h after the addition of TRAIL to HeLa cells (Fig. 8C). These analyses suggest that the IRES activity of the 5' UTR of PKC $\delta$  is enhanced under specific, rather than general, cellular conditions that reduce cap-dependent translation.

## DISCUSSION

PKC $\delta$  has been implicated in the response of cells to a myriad of signals, including those that promote differentiation, cell transformation, growth arrest, and apoptosis (8, 10, 25, 37, 43). While many studies have addressed the posttranslational activation and action of PKC $\delta$  in cells overexpressing native or dominant-negative forms of PKC $\delta$ , relatively little is known of the mechanisms that result in de novo PKC $\delta$  synthesis.

Most vertebrate transcripts contain 5' UTRs 20 to 100 nt long that are unlikely to impede translation initiation from the AUG codon (21). We report here that the 395-nt-long 5' UTR of PKC $\delta$  is potentially highly structured and has the ability to significantly repress the translation of a linked luciferase reporter gene with rabbit reticulocyte lysate as a source of translation factors. From this, we speculate that PKC $\delta$  might be translationally regulated in cells. In support of this, PKC $\delta$  was recently identified as a translationally regulated gene in a differential screen comparing resting and antigen-activated human T lymphocytes (34). An in vivo study suggests that PKC $\delta$  might be posttranscriptionally regulated in response to experimentally induced colitis in the rat as PKC $\delta$  protein levels increase without an accompanying increase in mRNA abundance (4). We also report here that the levels of PKC $\delta$  protein increase as HeLa cells grow to a high density, while its mRNA levels remain unchanged. This is consistent with other studies comparing PKC $\delta$  protein and mRNA levels in confluent and subconfluent cultures of RPTE and 3T6 cells (9, 27) and may thus represent a general feature of PKC $\delta$  function in response to cell density-dependent growth.

A subset of transcripts that encode growth factors, signaling molecules, and other genes involved in cell proliferation and apoptosis possess long, structured 5' UTRs that function as IRESs in a bicistronic assay (reviewed in reference 3). We provide evidence here that the 5' UTR of PKC $\delta$  acts as an IRES both in a monocistronic construct, where cap-dependent initiation is blocked by the introduction of a stable hairpin structure, and in a bicistronic construct. The monocistronic and bicistronic luciferase plasmids used in this study have previously been used to study the *c-myc* and Apaf-1 IRESs in a number of cell lines (6, 48, 49, 50). Compared to the control lacking an intergenic spacer, the IRES activity of the 5' UTRs of *c-myc*, Apaf-1, and PKC $\delta$  was greatest in HeLa cells and weakest in BALB/c cells, which supports the idea that some cell lines may lack noncanonical factors required for IRES function. However, we found that the IRES activity of the 5' UTR of PKC $\delta$  was comparable in MCF7 and HeLa cells, whereas MCF7 cells do not support the activity of the *c-myc* or Apaf-1 IRES (6, 50). This suggests that the utilization of cellular factors by different IRESs varies in particular cell types.

Cellular IRESs are considered likely to have an important function in maintaining synthesis of gene products when cap-

dependent translation is inhibited, such as during viral infection (18), hypoxia (47), apoptosis (16, 49), gamma irradiation (17), and amino acid starvation (11) and in the G<sub>2</sub>/M stage of the cell cycle (44). We show here that the IRES activity of the 5' UTR of PKC $\delta$  is greatest during TRAIL-induced apoptosis and when cells grow to a high density. A reduction in global protein synthesis and proteolytic cleavage of eIF4G is well documented during apoptosis (5, 30, 49). With increasing cell density of normal skin fibroblasts, eIF4E protein levels decrease and there is a concomitant increase in the unphosphorylated (eIF4E-sequestering) form of 4E-BP1, suggesting a loss of cap-dependent translation in densely growing cells (14). We also show here that the IRES activity of PKC $\delta$  is diminished in response to serum starvation, another condition known to reduce global protein synthesis and promote eIF4G degradation (5). These results suggest that the IRES activity of the 5' UTR of PKC $\delta$  is not enhanced under general conditions of cellular stress but is specifically enhanced in response to particular stimuli.

A cap-independent mechanism that would allow continued synthesis of PKC $\delta$  when cells grow to a high density and during apoptosis is consistent with PKC $\delta$  function under these conditions. Although an increase in PKC $\delta$  protein levels appears to be a general phenomenon of cells growing to a high density, its role here is only speculated. PKC $\delta$  may be required in densely growing cells to promote growth arrest and/or to prepare the cells for apoptosis. In support of this, many studies have implied a role for PKC $\delta$  in the induction of growth arrest (36, 43, 53) and loss of PKC $\delta$  is associated with cellular transformation and malignancy of gliomas (28, 29). In most apoptotic cells, PKC $\delta$  is activated by caspase 3-mediated proteolysis (10). The active proteolytic product of PKC $\delta$  is sufficient to induce apoptosis of HeLa cells (15). However, PKC $\delta$ -mediated apoptosis can occur in the absence of proteolytic cleavage (13). Furthermore, in response to mitochondrion-targeting toxins, a dominant-negative form of PKC $\delta$  inhibits caspase 3 activity, suggesting that PKC $\delta$  is also involved in a pathway leading to caspase 3 activation (31).

In apparent contrast to its role in growth arrest and apoptosis, PKC $\delta$  is also implicated in proliferative pathways. PKC $\delta$  promotes cellular transformation via the insulin-like growth factor I receptor (25), and elevated PKC $\delta$  levels are observed in simian virus 40-transformed rat fibroblasts and in highly metastatic mammary tumors (19, 26). Intriguingly, serum-stimulated PKC $\delta$  phosphorylates the repressor of eIF4E, 4E-BP1, in vivo and thus is involved in the activation of cap-dependent translation (23). This perhaps serves to highlight the important roles that PKC $\delta$  plays in many aspects of cell life, in promoting cell proliferation in response to some signals and in stimulating growth arrest and apoptosis in response to others. PKC $\delta$  may thus represent a switch for cell fate.

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