

# Parathyroid hormone-related peptide stimulates DNA synthesis and insulin secretion in pancreatic islets

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## Abstract

Parathyroid hormone (PTH)-related protein (PTHrP) is present in the pancreatic islet. Recent data in transgenic mice suggest that PTHrP might modulate islet mass and insulin secretion. In the present study, we assessed the effect of the N-terminal PTH-like region of PTHrP on DNA synthesis in isolated rat islets. PTHrP (1–34), between 1 pM and 10 nM, for 48 h stimulated [<sup>3</sup>H]thymidine incorporation into rat islets. This effect was maximally induced, about 2.5-fold over control, by 10 pM of this peptide, decreasing thereafter. In contrast, PTHrP (38–64) amide or PTHrP (107–139) were ineffective in increasing DNA synthesis in islets. Using reverse transcription followed by PCR, we confirmed that rat islets express PTHrP and the type I PTH/PTHrP receptor. Addition of a neutralizing anti-PTHrP antibody to the incubation medium of proliferating islets decreased islet

DNA synthesis by 30%. The effect of a submaximal dose (30 pM) of PTHrP (1–34) on DNA synthesis in rat islets was abolished by 25 nM bisindolylmaleimide I, a protein kinase C (PKC) inhibitor, but not by 25 μM adenosine 3',5'-cyclic monophosphothioate, Rp-isomer, a protein kinase A inhibitor. Moreover, 100 nM phorbol-12-myristate-13-acetate for 48 h also increased DNA synthesis 2-fold over controls in islets. PTHrP (1–34), at 100 nM, in contrast to 50 μM forskolin or 10 mM NaF, failed to affect adenylate cyclase activity in islet membranes. PTHrP, at 30 pM, was also found to increase 2-fold insulin released into the islet-conditioned medium within 24–48 h. Our results suggest that PTHrP is a modulator of pancreatic islet growth and/or function by a PKC-mediated mechanism.

*Journal of Endocrinology* (1999) **163**, 403–408

## Introduction

Parathyroid hormone-related protein (PTHrP) was initially identified as the main factor responsible for the paraneoplastic syndrome of humoral hypercalcemia of malignancy (Wysolmerski & Broadus 1994). The PTHrP gene has subsequently been detected in a broad variety of normal tissues, including the pancreatic islet (Drucker *et al.* 1989, Asa *et al.* 1990, Gaich *et al.* 1993, Philbrick *et al.* 1996). In addition, genes for both the common type I PTH/PTHrP receptor (PTHR1) cloned in bone and renal cells and a PTH-specific receptor are present in the pancreas (Abou-Samra *et al.* 1992, Usdin *et al.* 1995). Using radiolabeled binding techniques, PTHrP receptors having low affinity compared with the PTHR1, have been found in cultured rat insulinoma (RIN) cells (Gaich *et al.* 1993). These cells have no cAMP response to PTHrP (1–36) but do display a rapid and sensitive intracellular calcium response to either this peptide or PTH (1–34) (Gaich *et al.* 1993). Thus, a high-affinity PTH/PTHrP receptor associated with intracellular calcium transience,

undetected by using binding techniques, is likely to be present in RIN cells. Collectively, current findings indicate that various receptor types recognizing PTHrP and/or PTH are present in the pancreas.

Treatment of RIN cells with the islet differentiation agent sodium butyrate rapidly induces the transcription of the PTHrP gene (Streutker & Drucker 1991). In addition, recent studies in transgenic mice overexpressing PTHrP in the pancreatic β cells have shown lower fasting and postprandial plasma glucose, and higher plasma insulin concentration, compared with those of their normal littermates (Vasavada *et al.* 1996, Porter *et al.* 1998). Furthermore, insulin production was increased in the pancreas of these transgenic animals, as a result of an increased islet mass (Vasavada *et al.* 1996, Porter *et al.* 1998). Thus, PTHrP might act as an auto/paracrine factor in the pancreatic islet, as it does in a variety of other tissues (Philbrick *et al.* 1996).

In the present study, we found that PTHrP (1–34) stimulates DNA synthesis in isolated islets from adult rats, expressing PTHrP and the PTHR1, apparently by

a protein kinase C (PKC)-mediated mechanism. In addition, this effect appears to be associated with an increased insulin release into the islet incubation medium.

## Materials and Methods

### Reagents

Collagenase P from *Clostridium histolyticum* was from Boehringer (Mannheim, Germany). Forskolin, 3-isobutyl-1-methylxanthine (IBMX), NaF, human PTHrP (1–34) and human PTHrP (107–139) were from Sigma (St Louis, MO, USA). Human PTHrP (38–64) amide was from Peninsula (Belmont, CA, USA). Fetal calf serum (FCS) was from Biochrom KG (Berlin, Germany). Adenosine 3',5'-cyclic monophosphorothioate, Rp-isomer (Rp-cAMPS) was supplied by Biolog Life Science Institute (Bremen, Germany). Bisindolylmaleimide I (BIM) and phorbol-12-myristate-13-acetate (PMA) were from Calbiochem (San Diego, CA, USA). Rat insulin was from Linco (St Charles, MO, USA). Ham's F10 medium was from Seromed (Biochrom KG).

### Pancreatic islets

Pancreatic islets were isolated from normal female Wistar rats (200–250 g body weight) by collagenase digestion, as previously described (Malaisse-Lagae & Malaisse 1984).

### Pancreatic islet plasma membranes

A group of 800 islets was homogenized in a Potter homogenizer (ten strokes), in 100 µl 25 mM Tris-HCl, pH 7.5, containing 5 mM MgCl<sub>2</sub>, 0.6 mM EGTA and 1.1 mM dithiothreitol, at 4 °C. The resulting debris was separated by sedimentation at 100 g, at 4 °C, and discarded; the supernatant, containing plasma membranes, was used to determine its adenylate cyclase activity.

### DNA synthesis

Groups of 50 islets were cultured, under sterile conditions, in Ham's F10 medium (6.1 mM D-glucose) with 1% FCS and antibiotics, in the absence (control) or presence of either different PTHrP peptides or 100 nM PMA, with or without 25 nM BIM, a PKC inhibitor (Toullec *et al.* 1991), or 25 µM Rp-cAMPS, a protein kinase A (PKA) inhibitor (Wang *et al.* 1992), for 24 h at 37 °C; the two latter agonists were added 1 h before PTHrP (1–34). The islets were then further cultured for 24 h in fresh medium containing the same agents and 1.5 µCi/ml [<sup>3</sup>H]thymidine (87 Ci/mmol, Amersham International plc, Amersham, Bucks, UK). Islet DNA was then precipitated with 4% HClO<sub>4</sub> at 4 °C, separated by filtration (Glass Microfiber Filters GF/C, Whatman, Maidstone, Kent, UK), and

β-counted (Gallo *et al.* 1986). In parallel experiments, [<sup>3</sup>H]thymidine was not added, and islet function was studied at the end of the 48 h incubation period, by measuring the insulin response to D-glucose (2.8 and 16.7 mM) for 60 min (Malaisse-Lagae & Malaisse 1984). After incubations, the islet-conditioned medium was collected, and stored at –20 °C for insulin determination. In order to evaluate the effect of anti-PTHrP antibodies on islet cell proliferation, islets were incubated for 48 h in culture medium with 1–10% FCS, and 7 µg/ml of either affinity-purified anti-PTHrP neutralizing antibody C7 or nonimmunogenic rabbit IgG (Valín *et al.* 1997), and [<sup>3</sup>H]thymidine incorporation into islet DNA was measured as described above.

### Adenylate cyclase activity

Adenylate cyclase activity was determined in isolated islet plasma membranes, as described (Valverde *et al.* 1979). Aliquot samples (40 µl) of the plasma membrane preparations (1.4–1.9 µg protein (Lowry *et al.* 1951)) were added to 35 µl of a reaction mixture containing 70 µM ATP, 5 mM MgCl<sub>2</sub>, 25 mM Tris-HCl and 0.5 mM IBMX, without (control) or with 100 nM PTHrP (1–34), 50 µM forskolin or 10 mM NaF. After 60 min at 37 °C, the reaction was stopped with ethanol (final concentration 65%). After centrifugation at 1700 g for 15 min, at 4 °C, aliquot samples (365 µl) of the supernatants were vacuum-dried, and then resuspended in 120 µl of assay buffer for cAMP determination (RIANEN cAMP; Dupont, Brussels, Belgium).

### RNA extraction and reverse transcription-PCR (RT-PCR)

Total RNA was extracted from rat islets using guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski & Sacchi 1987). Total RNA aliquots were added to a reaction mixture containing 1 mM MgSO<sub>4</sub>, 0.2 mM of each dNTP, 0.1 U/µl avian myeloblastosis virus (AMV) reverse transcriptase, 0.1 U/µl thermostable DNA polymerase from *Thermus flavus* (Access RT-PCR System; Promega, Madison, WI, USA), and 1 µM specific primers: 5'-TGCAGCGGAGACTGGTTCAG-3' (sense) and 5'-CCTCGTCTGTGACCCAAA-3' (antisense) (PTHrP); and 5'-GATCGGACGATGTCTTTACC-3' (sense) and 5'-GGCGGTCAAATACCTCC-3' (antisense) (PTHR1).

Using these primers, PCR amplification yields products of 301 bp (PTHrP) or 483 bp (PTHR1) (Li *et al.* 1996, García-Ocaña *et al.* 1998). Total RNA and the primers were preincubated for 5 min at 65 °C. Then the reaction mixture was incubated for 45 min at 48 °C, and 2 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 58 °C, and 2 min at 68 °C, with a final extension of 7 min at 68 °C. Negative controls without AMV transcriptase were usually included in our RT-PCR system, and

amplification of glyceraldehyde 3-phosphate dehydrogenase, as a constitutive gene control, with specific primers was also performed (García-Ocaña *et al.* 1998, Largo *et al.* 1999). In addition, total RNA from rat kidney cortex, containing PTHrP and PTHR1 mRNAs (Largo *et al.* 1999), was also coamplified using the same primers described above, as a positive control. The PCR products were separated on 1.5% agarose gels, and bands were visualized by ethidium bromide staining. The specific PCR products were purified by adsorption to silica (GeneClean Bio 101, La Jolla, CA, USA), and subsequently reamplified using the same protocol described above, using 30 cycles. The identities of the PCR products were confirmed by sequencing with a dye-terminator cycle-sequencing kit (Perkin-Elmer, Branchburg, NJ, USA), using Taq FS DNA polymerase. Sequences were resolved on an ABI PRISM 377 automatic sequencer (Perkin-Elmer).

*Insulin release*

Insulin was measured in the islet incubation medium by RIA, using rat insulin as standard, and a guinea-pig anti-insulin serum developed in our laboratory, as previously described (Valverde *et al.* 1988).

*Statistical analysis*

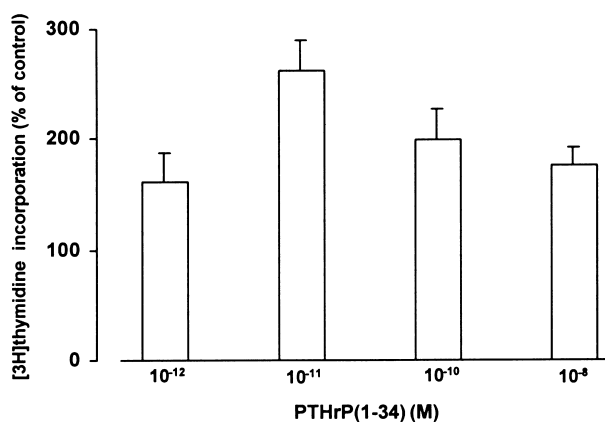
Results are expressed as mean ± s.e.m. (number of observations). Statistical significance, reported as  $P < 0.05$ , was assessed by unpaired *t*-test.

**Results**

PTHrP(1–34), from 1 pM to 10 nM, for 48 h was found to increase [<sup>3</sup>H]thymidine incorporation into rat islets in medium with 6.1 mM glucose (Fig. 1). This effect was maximal at 10 pM PTHrP (1–34), decreasing thereafter (Fig. 1). In contrast, PTHrP (38–64) and PTHrP (107–139), both at 10 pM, failed to stimulate islet DNA synthesis for the same time period.

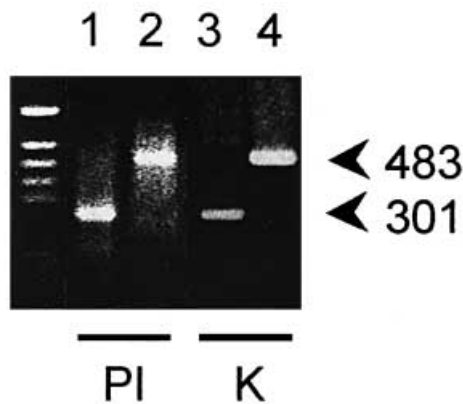
We obtained PCR products in rat islets identical to those found in rat kidney, indicating the presence of PTHrP and PTHR1 mRNA (Fig. 2). In order to assess a possible autocrine effect of PTHrP on pancreatic islets, we added an anti-PTHrP antibody to the islet incubation medium in the presence of 10% FCS. The presence of this antibody significantly decreased the 10% FCS-stimulated [<sup>3</sup>H]thymidine incorporation into islets from  $398 \pm 36$  d.p.m. to  $276 \pm 69$  d.p.m. ( $P < 0.025$ ); while it was  $407 \pm 15$  d.p.m., in the presence of nonimmunogenic rabbit IgG, compared with  $159 \pm 32$  d.p.m. in the 1% FCS control ( $n = 4$ ,  $P < 0.01$ ), after 48 h incubation.

We also evaluated the putative role of PKC and PKA in the PTHrP (1–34)-stimulatory effect on DNA synthesis

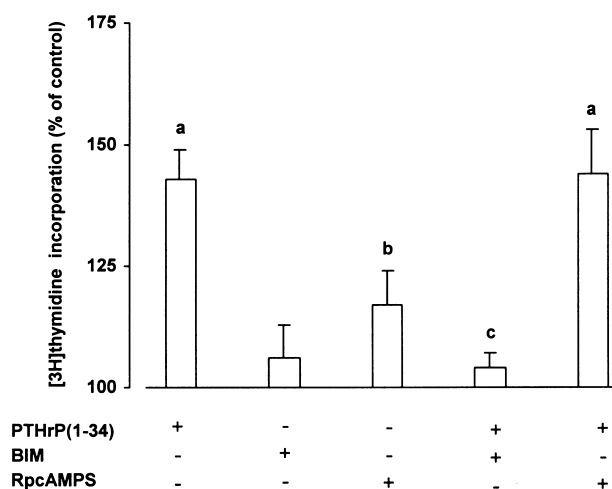


**Figure 1** Dose-dependent effect of PTHrP (1–34) on [<sup>3</sup>H]thymidine incorporation into DNA in isolated rat islets after 48 h stimulation. Values are mean ± s.e.m. of four to nine measurements.  $P < 0.05$  or lower, at each PTHrP (1–34) dose tested, compared with nonstimulated control (100%, or  $107 \pm 29$  d.p.m./tube).

into rat islets. We found that 25 nM BIM, a PKC inhibitor (Toullec *et al.* 1991), in contrast to 25 μM RpcAMPS, a PKA inhibitor (Wang *et al.* 1992), abolished this effect induced by a submaximal dose (30 pM) of PTHrP (1–34) (Fig. 3); neither BIM nor RpcAMPS, when tested alone, had any significant effect on islet DNA synthesis (Fig. 3). The inhibitory effect of BIM on the stimulated DNA synthesis induced by PTHrP (1–34) was not associated with an impaired islet function, since preincubation with BIM for 48 h did not significantly affect the subsequent islet insulin secretion for 1 h, which was:  $146 \pm 6\%$  (without BIM) and  $136 \pm 1\%$  (with BIM) in the presence of 16.7 mM glucose, compared with the corresponding control values in the presence of 2.8 mM glucose (100%):



**Figure 2** PTHrP (lanes 1 and 3) and PTHR1 (lanes 2 and 4) mRNA in rat pancreatic islets (PI) and rat kidney (K). RT-PCR was carried out with specific primers, and the corresponding PCR products were reamplified, using the Access RT-PCR System (Promega). PCR products were electrophoresed on 1.5% agarose gel. DNA markers are shown in the first column on the left.



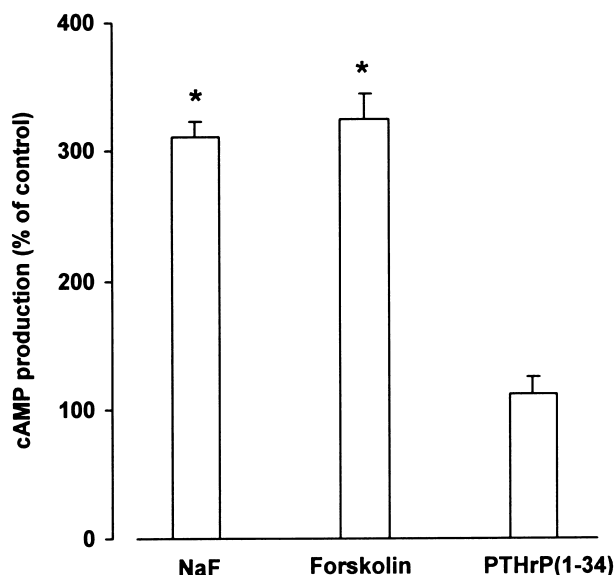
**Figure 3** Effect of 25 nM BIM (PKC inhibitor) and 25  $\mu$ M RpcAMPS (PKA inhibitor) on the DNA synthesis-stimulatory effect of 30 pM PTHrP (1-34) in isolated rat islets for 48 h. The inhibitors were added 1 h before PTHrP (1-34) addition. Values are mean  $\pm$  S.E.M. of four to six measurements. a,  $P < 0.01$ , compared with nonstimulated control (100%, or  $265 \pm 44$  d.p.m./tube); b,  $P < 0.01$ , compared with RpcAMPS+PTHrP (1-34); c,  $P < 0.001$ , compared with PTHrP 1-34) alone.

$362 \pm 36$  pg/islet (without BIM) or  $475 \pm 51$  pg/islet (with BIM) (mean  $\pm$  S.E.M., corresponding to two independent experiments performed in six replicates). Furthermore, 100 nM PMA, a PKC stimulator (Gagnon *et al.* 1993), also significantly stimulated islet DNA synthesis ( $190 \pm 22\%$  of control;  $n = 4$ ,  $P < 0.05$ ). PTHrP (1-34), at 100 nM, failed to affect adenylate cyclase, while either 50  $\mu$ M forskolin or 10 mM NaF stimulated about 3-fold over control this enzymatic activity, in rat islet membranes (Fig. 4).

PTHrP (1-34), at 30 pM, induced an increase of insulin released into the islet culture medium after both subsequent 24 h time periods, which was:  $279 \pm 33$  and  $198 \pm 29\%$  respectively, of control ( $174 \pm 20$  pg/islet) ( $n = 4$ ,  $P < 0.05$ ).

## Discussion

In the present study, we found that both N-terminal PTHrP and PTH, at pM doses, stimulate DNA synthesis in isolated rat islets. In contrast, other middle and C-terminal PTHrP fragments failed to affect DNA synthesis in islets. Consistent with previous findings, using immunohistochemistry and/or Northern blot analysis, in human and rat islets (Drucker *et al.* 1989, Asa *et al.* 1990, Gaich *et al.* 1993, Usdin *et al.* 1995), we have detected PTHrP and PTHR1 mRNA, by RT-PCR, in adult rat islets. In addition, we found that the presence of an anti-PTHrP neutralizing antibody decreased rat islet DNA synthesis. A recent study in islets isolated from neonatal



**Figure 4** Effect of 100 nM PTHrP (1-34), 10 mM NaF, and 50  $\mu$ M forskolin, on adenylate cyclase activity in isolated rat islet plasma membranes. Islet membranes were obtained from homogenized islets after centrifugation at 100 g. Adenylate cyclase activity was determined in aliquot samples of the supernatant incubated in the presence of ATP and the agonists for 1 h at 37 °C. Produced cAMP was measured by RIA. Values are mean  $\pm$  S.E.M. of three to seven experiments. \* $P < 0.001$ , compared with nonstimulated control.

rats failed to find a significant increase in islet proliferation rate, estimated as percent bromodeoxyuridine-positive nuclei, after continuous treatment with different PTHrP peptides (Porter *et al.* 1998). The reason for these discrepancies is unknown. However, using islets from adult rats, and [ $^3$ H]thymidine incorporation to assess islet proliferation, we observed that higher PTHrP (1-34) concentrations, in the range of those tested in the aforementioned study (Porter *et al.* 1998), were less stimulatory of islet DNA synthesis than lower doses.

Previous reports in mice overexpressing PTHrP in the pancreatic islets demonstrated an increased islet mass associated with islet hyperplasia (Vasavada *et al.* 1996, Porter *et al.* 1998). These animals, opposite to PTHrP-overproducing tumor hosts, had neither hypercalcemia nor high plasma PTHrP (Wysolmerski & Broadus 1994, Vasavada *et al.* 1996). These *in vivo* studies and the *in vitro* results herein support the concept that PTHrP in the pancreatic islets is an autocrine factor inducing islet proliferation.

Our results suggest that the effect of PTHrP (1-34) on islet DNA synthesis depends on PKC activation. In this regard, PTHrP (1-34), at pM concentration, has been shown to induce a peak of PKC activation in other cells (Gagnon *et al.* 1993, Whitfield *et al.* 1994). Moreover, PKC activation appears to be a mediator for the mitogenic effects

of the N-terminal region of PTHrP and PTH in various cell types (Sömjen *et al.* 1990, Jouishomme *et al.* 1994, García-Ocaña *et al.* 1995).

We found that, in contrast to the common pattern of signaling through PTHR1, PTHrP (1–34) failed to increase cAMP in rat islets. This is consistent with previous findings on RIN cells displaying a cytosolic calcium response, but not a cAMP response, to PTHrP (1–36), detectable at 1 pM (Gaich *et al.* 1993). High-affinity binding sites for this peptide were not detected in these cells (Gaich *et al.* 1993). In this regard, the presence of a sensitive intracellular calcium response in the absence of high-affinity binding sites for N-terminal PTHrP has also been reported to occur in squamous carcinoma cells and keratinocytes (Orloff *et al.* 1992). Recent studies suggest that these cells appear to express a PTH/PTHrP receptor different from, but partially homologous to, the PTHR1 (Orloff *et al.* 1995). Whether such a receptor is present in both RIN cells and rat islets or, alternatively, PTHR1 are of low abundance in these cells, and then undetected by radiolabeled binding techniques but revealed by mRNA analysis, deserves further studies.

PTHrP (38–64) amide was found to be ineffective in altering islet DNA synthesis. Recently, it has been demonstrated that PTHrP (38–94) amide, a PTHrP fragment produced and secreted by RIN cells, is unable to stimulate adenylate cyclase but induces cytosolic calcium increments in these cells (Wu *et al.* 1996). This effect is thought to be mediated by a PTHrP (67–86)-specific receptor similar to that identified in squamous carcinoma cells (Orloff *et al.* 1996). Our data do not allow us to conclude that such a receptor is present in pancreatic islets.

A recent report has shown that the insulin response to glucose of perfused islets from PTHrP-overexpressing mice is similar to that of normal islets (Vasavada *et al.* 1996). In contrast, Fadda *et al.* (1990) previously reported that PTH (1–34), in the  $\mu\text{M}$  range, triggered a rapid increase in glucose-induced insulin release by adult rat islets. This effect appeared to depend on a rise in cytosolic calcium, leading to a secondary PKC activation (Fadda *et al.* 1990). Our data herein are consistent with the latter findings, since the stimulatory effect of PTHrP (1–34) on islet DNA synthesis was found to be associated with an increased insulin secretion into the islet culture medium.

PTHrP does not appear to be a developmental factor in the islet, since homozygous PTHrP knockout mice have anatomically normal islets at birth (Karaplis *et al.* 1994). However, taken together, the bulk of previous studies and the present one support the concept that PTHrP is involved in the regulation of postnatal islet growth and/or function.

## Acknowledgements

This work was supported by grants from Dirección General de Investigación Científica y Técnica (DGICYT,

95/0048) and Fondo de Investigación Sanitaria (FIS, 96/1383, 96/1167 and 98/1230). We are grateful to Dr A Díaz (DNA Automatic Sequencer Laboratory, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Madrid) for sequencing the PCR products, and to Dr R Bosch (Department of Physiology, Alcalá University, Madrid) for providing us with the primers for PTHR1. J C and A V are research fellows of Fundación Conchita Rábago. A R and F M are research fellows of Ministerio de Educación y Cultura and Comunidad Autónoma de Madrid respectively. We also thank E Martín-Crespo for skillful technical assistance.

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Received 10 March 1999

Revised manuscript received 30 June 1999

Accepted 22 July 1999