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Expression of different mitogen-regulated protein/proliferin mRNAs in Ehrlich carcinoma cells

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Abstract

Results from in vivo and from serum-free primary cultures of Ehrlich cells suggest that the expression of mitogen-regulated protein/proliferin (MRP/PLF) mRNAs is not essential for proliferation of this murine tumor. Two sizes for MRP/PRL-related open reading frames (ORFs) have been detected by reverse transcription/PCR amplification. They are almost identical to that reported for PLF-1; but 20% of the amplified cDNA included a shorter ORF, which lacks the entire sequence corresponding to that of the exon 3 of the *mrp/plf* genes. Ehrlich carcinoma may represent a good model to study regulation of expression and physiological roles of MRP/PLFs in vivo.

Key words: Proliferin; Mitogen-regulated protein; Ehrlich carcinoma

1. Introduction

Proliferins (PLFs) are members of the growth hormone/prolactin (GH/PRL) family [1] identified with mitogen-regulated proteins (MRPs) [2]. They are expressed and secreted by different murine cultured cell lines and trophoblastic giant cells in vivo [2,3]. The mature proteins are glycopeptides which bind to the cation-independent mannose-phosphate receptor [4], and to a second receptor detected in placenta, uterus and mammary gland of pregnant mice [5]. The involvement of MPR/PLF expression in proliferation, differentiation, immortalization and transformation of several mouse embryo cultured cells have also been studied [6–8]. Nevertheless, the expression of MRP/PLF does not always correlate with cell proliferation and physiological roles of these proteins have not been completely ascertained, being autocrine and paracrine functions hypothesized for these proteins [1–8]. Mouse chromosome 13 contains approximately six copies of *mrp/plf* genes (as well as prolactin and placental lactogen genes [9]. All the members of the *GH/PRL* family are derived from a common precursor and have similar intron/exon boundaries; three *mrp/plf* promoters are known so far to have slight differences among them [10,11]. In addition, other proliferin-related transcripts are detected in mouse placenta, which follow a different time course than the initially observed for proliferin [12]. Transcriptional and post-transcriptional mechanisms are involved to control the MRP/PLF expression [11,13–15]. Three different MRP/PLF sequences have been deduced (PLF-1, PLF-2, MRP/PLF-3) [10], some authors doubt that the physiological roles of each gene product are identical [2,5,6,13].

Using a PLF-2 cDNA from BALB/c mouse placenta as a probe [16], hybridizable mRNA levels have been determined during Ehrlich carcinoma progression in vivo and primary cultures. By reverse transcription/polymerase chain reaction (RT/PCR) experiments, we have detected that two different PLF-1 open reading frames (ORFs) are expressed by these cells. Results suggest that expression of MRP/PLF is not related to tumor proliferation in this murine cancer model.

2. Materials and methods

2.1. Cell cultures

A hyperdiploid Lettré strain of Ehrlich ascitic carcinoma was maintained in 2-month-old Swiss mice, as reported previously [17]. The growth curve for the tumor has also been described elsewhere [18]; life span of the animals is 16 ± 1 days. On different days, cells were harvested, washed twice and frozen in sterile conditions for RNA extraction. Primary cultures of Ehrlich cells were carried out in Dulbecco's Modified Eagle's medium: Ham's F12 medium (1:1) supplemented with 0.2% bovine serum albumin and antibiotics as described previously [19].

2.2. Northern blot analysis

Total RNA (10 μg) isolated by the acid guanidinium–phenol–chloroform method, was fractionated by formaldehyde-agarose gel electrophoresis and transferred to Nylon membranes [19,20]. The transferred RNA was hybridized to a proliferin cDNA from mouse placenta containing the full-length open reading frame for the PLF-2 sequence [16]. Filters were rehybridized with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.1.1.8) and ornithine decarboxylase (ODC, EC 4.1.1.17) probes described previously [12]. GAPDH mRNA (a housekeeping gene product) was detected as an internal control; ODC mRNA was used as a poorly translated and proliferation-related message [19]. Probes were labeled by Multiprime DNA Labeling System with [α-^{32}P]dCTP (Amersham Iberica, Spain). Each experiment was repeated for several times from different cell batches, and similar results were obtained. Representative results are shown in figures. Autoradiographs were analyzed by transmittance densitometry using a Hoefer

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GS 300 scanning densitometer and the GS370 1-D Electrophoresis Data System version 2.3 (Hoefer Scientific, USA).

2.3. Polysome isolation and fractionation

Ehrlich carcinoma cells were harvested, washed twice in the phosphate-buffered saline [17], and lysed in 10 mM HEPES, 1.5 mM magnesium acetate, 10 mM KCl, 7 mM β-mercaptoethanol, 100 μg/ml cycloheximide, 50 U/ml ribonuclease inhibitor from human placenta, 1% Nonidet P-40 and 1% deoxycholate (50×10^6 cells/1.5 ml, 15 min, pH 7.5, 4°C). After centrifugation (8,000 rpm, 10 min, 4°C), supernatants (1 ml) were layered onto a 10–40% (w/v) linear sucrose gradient prepared in 10 mM HEPES, 50 mM magnesium acetate, 0.5 M KCl, 7 mM β-mercaptoethanol, 100 μg/ml cycloheximide and centrifuged again at $\omega^2 t = 9.02 \times 10^{10}$ rad^2/s in a Beckman SW 28 rotor at 5°C. Then, 1.5 ml fractions were collected and absorbance was measured at 260 nm. Thereafter, three consecutive fractions were phenol extracted, precipitated with ethanol, dried and solved in equal volumes of diethylpyrocarbonate-treated water. Samples were divided in two aliquots and parallel electrophoresed as described above. From each sample, an aliquot was transferred, baked and hybridized, the other one was used to confirm the integrity of RNA (based on appearance of ribosomal RNA bands).

2.4. RT/PCR amplification and sequencing

Samples of mRNA were obtained using Mini Fast Track System (EcoGen, UK). M-MuLV reverse transcriptase (Boehringer Mannheim, Germany) and AmpliTaq DNA polymerase (Perkin Elmer-Cetus, USA) were used. The basic protocol has been described by Rolfs et al. [21]. Primers were designed to be complementary to reported genomic and cDNA sequences for MRP/PLFs [1,2,4,7]: primer 1, from 63 to 47 bases upstream of the translation initiation codon (5′-TTCCAACTCCAGTIAAG-3′); primer 2, from 18 to 5 bases downstream the stop codon plus a *Pst*I site (5′-AGCTGCAGAGCACATGAAAGA-3′). Amplification was allowed for 22 cycles: 93°C for 1 min, 55°C for 30 s, 72°C for 1 min (final extension 10 min); after 22 amplification cycles, the number of initial templates is expected to be exponentially-related to the observed product amount [5,21]. For Southern blotting, Nytran (Schleicher and Schuell, Germany) and the PLF-2 probe described above was used. There is a *Pst*I internal site at 8–3 bp upstream the translation initiation codon in every MRP/PLF sequence known so far; thus, RT/PCR products from two different cell sample mRNAs (A and B) were purified from agarose gel by the Gene Clean method, digested with *Pst*I and subcloned using *Pst*I-digested dephosphorylated pGEM3Z vector (Promega, USA). Both strand sequencing was performed as previously reported [20] and using an ALF DNA sequencer (Pharmacia, Sweden).

3. Results and discussion

Serum, several growth factors and virus-mediated oncogenic transformation induce PLF-1 expression in 3T3 fibroblasts, reaching a peak at the onset of DNA synthesis [1]; maximum PLF-2 expression in mouse placenta also occurs during placental growth [3,4,16]. Nevertheless, MRP/PLFs do not seem to be essential factors for proliferation of every cultured mouse embryonic cell line [2,7]. Krebs tumor cells growing in vivo also express PLF-1 on day 10 after transplantation [1]. However, as far as we know, the PLF expression along tumor progression in vivo has not been studied. Fig. 1 shows results of Northern blot experiments, using samples collected on different days after Ehrlich carcinoma transplantation. A PLF-hybridizable mRNA is detected from day 8 onwards, reaching the maximum level on day 12, when the tumor has reached the plateau phase of growth. The PLF-2 probe recognized a smear band of about 1 kb, as reported for MRP/PLF messages detected in different embryonic cell lines [1,7,16]. Working with serum stimulated BALB/c 3T3 fibroblasts, Linzer and Wilder [14] suggest that this heterogeneity can be due to post-transcriptional events (i.e. different poly(A) tail lengths). No hybridization signal could be detected by the PLF-2

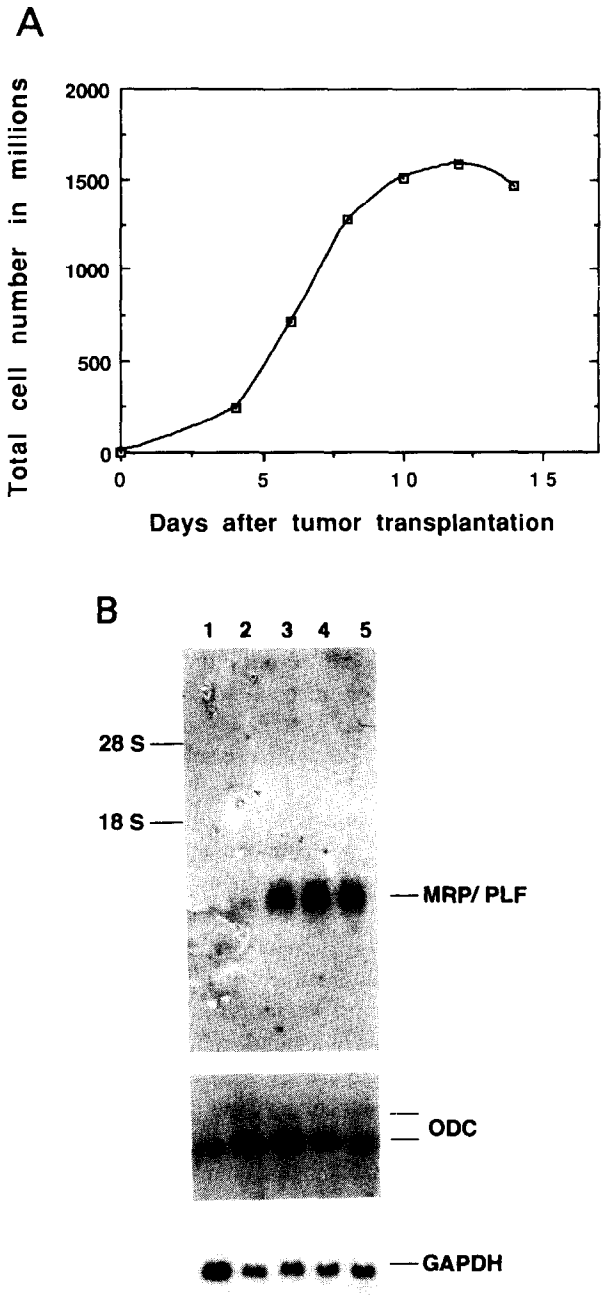

Fig. 1. Evolution of the PLF mRNA levels during in vivo progression of Ehrlich ascitic tumor. (A) Total cell numbers obtained during tumor progression in vivo on different days after tumor inoculation (5×10^6 cells/mouse). (B) Northern analysis of RNA from the Ehrlich cells shown in (A) on day 6 (lane 1), day 8 (lane 2), day 10 (lane 3), day 12 (lane 4) and day 14 (lane 5) after tumor transplantation. A single membrane was hybridized to cDNA probes for PLF, GAPDH and ODC. Relative hybridization signals for PLF mRNA normalized to those of GAPDH were: 0 (days 4 and 6), 0.16 (day 8), 0.72 (day 10), 1.00 (day 12), 0.90 (day 14).

probe even after longer exposure of filters containing RNA samples from cells harvested on day 4 and day 6 after the Ehrlich carcinoma transplantation, when the tumor is in the exponential phase of growth (Fig. 1A and [18]). In contrast to the results obtained with BALB/c 3T3 fibroblasts [1] or mouse placenta [16], high molecular size MRP/PLF mRNA precursors were not detectable either at any tested condition. The signals obtained after rehybridization of the filters with an internal control probe (GAPDH) and with a specific ODC probe is also shown for comparison. As can be observed in Fig. 1B, ODC and MRP/PLF mRNAs follow a different time-course throughout the Ehrlich tumor progression.

The relative distribution of free messenger and messenger associated with high density polysomes give a validated indication of the actual translatability of an mRNA [22]. In Fig. 2A, a representative result of the PLF 2-hybridizable mRNA distribution on isolated polysome fractions from Ehrlich cells harvested at plateau phase of growth is shown: most of the signal intensity was associated with polysome fractions (> 150 S) and less than 20% of the signal was detected as free mRNA. GAPDH and ODC mRNAs were also detected on the same filters, both messages are longer in size than MRP/PLF mRNA; however, they appeared in a major extent as free mRNAs or associated to a lower number of ribosomes (Fig. 2B). The poorly translated ODC mRNAs [19] and the MRP/PLF-related mRNAs exhibited opposite distribution patterns on polysome profiles from resting Ehrlich carcinoma cells. Results suggest that Ehrlich carcinoma actively translates proliferin-related mRNAs at plateau phase of growth in vivo.

Ehrlich carcinoma cells are able to proliferate in the absence of serum. The possibility of a relationship between MRP/PLF expression and cell proliferation in the absence of the host interferences was tested on serum-free Ehrlich cell cultures. The seeded cells had been harvested on day 6 (exponential phase of growth) or on day 10 (plateau-phase of growth) after tumor transplantation. At different times, cultured samples were obtained and PLF 2-hybridizable mRNA levels were detected by Northern blotting at different times. As observed in Fig. 3, MRP/PLF-related mRNA levels were not increased during proliferation of primary serum-free cultures of Ehrlich cells when seeded cells had been harvested at plateau-phase of growth in vivo. In addition, no PLF 2-hybridizable mRNA could be detected in serum-free cultures when seeded cells came from day 6 Ehrlich cells growing in vivo. Therefore, autocrine factors do not seem to have any effect inducing the MRP/PLF expression in our model; and in agreement with the results observed in vivo, proliferin expression does not seem to be essential for Ehrlich cell growth, as also reported for other murine embryonic cells [2,7].

As stated above, several copies of *mrp/plf* genes are present in the mouse genome. Once the evolution of

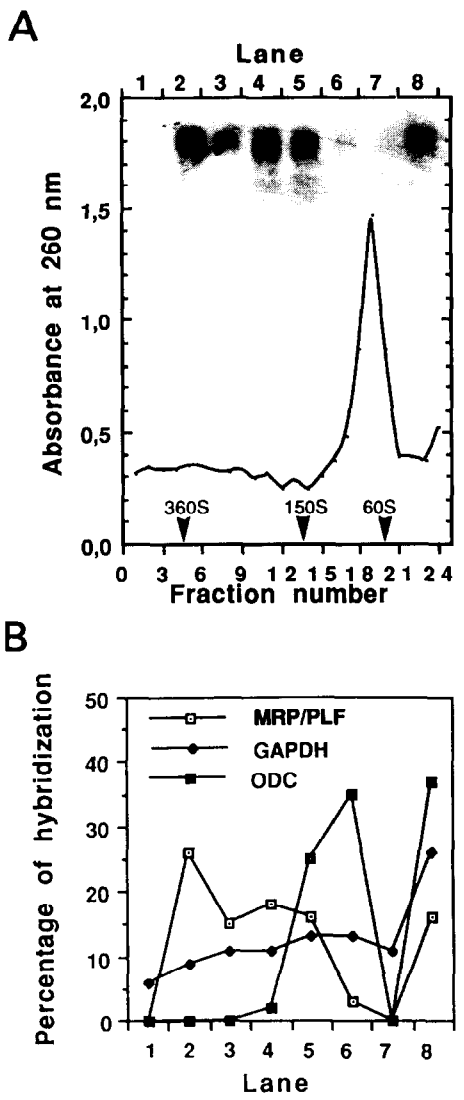

Fig. 2. Distribution of PLF mRNAs in polysome profiles from Ehrlich cells in plateau phase of growth. (A) Absorbance profiles measured at 260 nm on individual fractions (1.5 ml). Arrows indicate sedimentation coefficients corresponding to the fractions at 5°C. Northern blot analysis of MRP/PLF mRNA from pooled fractions; lane 1 (fractions 1–3), lane 2 (fractions 4–6), lane 3 (fractions 7–9), lane 4 (fractions 10–12), lane 5 (fractions 13–15), lane 6 (fractions 16–18), lane 7 (fractions 19–21), lane 8 (fractions 22–24) is also shown on the profile. (B) Distribution of the hybridization signal intensities for MRP/PLF, GAPDH and ODC mRNAs on the same filter. The sum of the signal intensities for each mRNA was considered 100%.

PLF-2-hybridable RNA levels had been observed in Ehrlich cells growing in vivo and in vitro, the following aim was to determine the similarity between the PLF expressed by Ehrlich cells and the MRP/PLF sequences deduced so far. Thus, mRNA samples from in vivo and cultured Ehrlich cells were used for RT/PCR experiments using primers designed to amplify the entire open reading frames encoding MRP/PLFs. After separation of the PCR products in agarose gels, two different bands were detected by ethidium bromide staining (Fig. 4A):

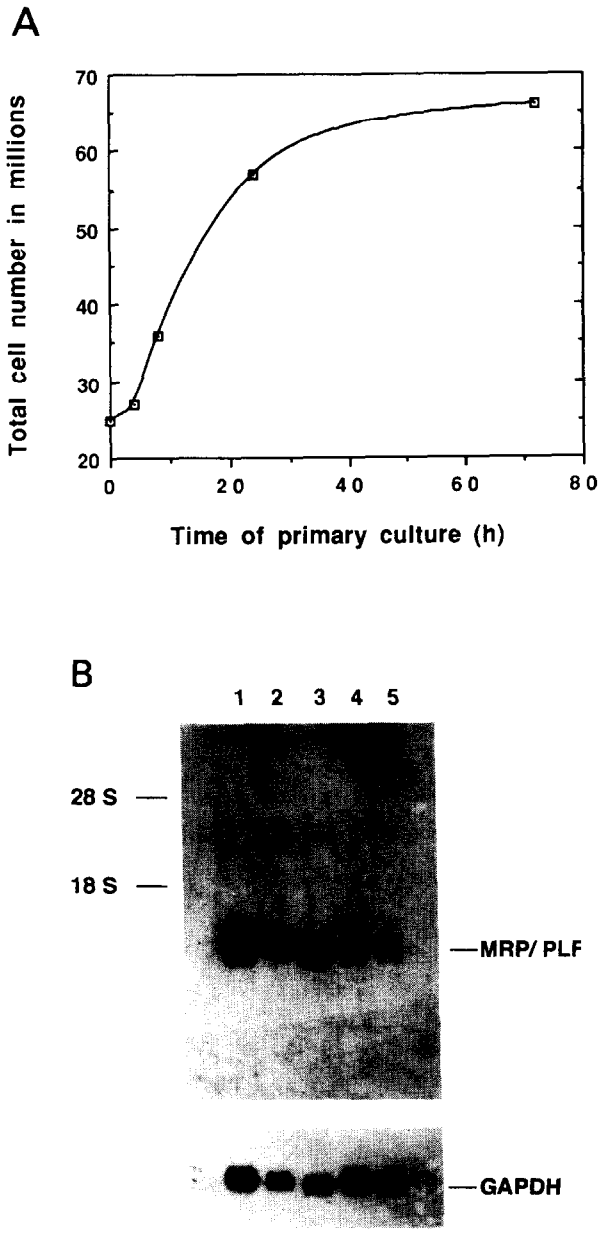

Fig. 3. Evolution of the PLF mRNA levels during primary cultures of Ehrlich tumor cells. (A) Total cell numbers obtained at different times after seeding of freshly harvested Ehrlich cells at plateau phase of growth (2.5×10^5 cells/ml). (B) Northern analysis of RNA from Ehrlich cells collected at different times during primary cultures: seeded cells (lane 1), hour 6 (lane 2), hour 12 (lane 3), hour 24 (lane 4), hour 48 (lane 5). A single membrane was hybridized to cDNA probes for PLF and GAPDH. Relative hybridization signals for PLF mRNA normalized to those of GAPDH were: 1 (hour 0), 0.62 (hour 6), 0.65 (hour 12), 0.21 (hour 24), 0.08 (hour 48).

the major band (about 80% after densitometric analysis) had the predicted size (about 760 bp) for amplification products containing the full PLF coding sequences. The minor band had a shorter size (about 650 bp), but was also recognized by the PLF-2 probe by Southern blotting (results not shown). The existence of two PLF-hybridizable messengers with different lengths could contribute to the smear band which is observed in Northern blots (Figs. 1–3).

After *Pst*I digestion and subcloning of the amplification products, three regular size-inserts (704 bp: PLF-AI, PLF-AII, PFL-BI) and two short size inserts (596 bp: sPLF-A and sPLF-B) were sequenced. The deduced polypeptide sequences encoded by PLF-AI and PLF-BI were identical to those of PLF-1 [1]. PLF-AII only presented three puntual base substitutions with respect to PLF-1 (Fig. 4B); Ala^{107} being the only PLF-2 characteristic residue [16] detected. These simple substitutions could either reflect real differences occurring in these hyperdiploid cells or have been introduced by polymerase during amplification. The differences among the three MRP/PLF sequences deduced so far are also caused by substitutions of 3 (MRP/PLF 3) or 5 (PLF-2) puntual bases with respect to the PLF-1 cDNA sequence [10,16]. The deduced sequences encoded by the short inserts (Accession Number in EMBL Data Bank X-75557) are also shown in Fig. 4B; they also correspond to PLF-1 sequences but lack the entire sequence encoding for amino acid residues 70–105, that are present in every MRP/PLF deduced so far. Hence, the translation product of these short mRNAs (sPLF) would lack two potential glycosylation sites and one of the most turn-enriched hydrophilic fragments present in MRP/PLFs. This delection could bring together two α-helix segments predicted for rodent prolactin-like proteins [12]. In addition, Cys^{87} (Cys^{58} in the mature PLFs), which is a conserved residue among every member of the mouse GH/PRL family [12,23] would be absent in the translation product of sPLF mRNAs. This Cys residue seems to be involved in disulphide bonds in MPR/PLFs and other members of the family [23]. These changes should lead to significant variations in the sPLF properties for macromolecular interaction with respect to other reported MRP/PLFs.

On the other hand, the deleted residues encoding for amino acids 70–105 correspond exactly to those of the exon 3 of a *mrp/plf* genomic clone named *mrp/plf* 3 by Connor et al. [10]. The genomic organization of the other *mrp/plf* genes seems to be similar [5,10]. It is noteworthy, that the beginning of the reported exon 3, is also the point in which a major gap is observed when alignment is carried out among placental proliferin-related protein (PRP) and the other members of the GH/PRL family [12]. Altered forms of other members of this family have been reported; for instance, the 22 and 20 kDa forms of the pituitary human GH are products of an alternative splicing of a human *GH* gene, the 20 kDa GH being about 10% of the synthetized hormone [24]. The high degree of identity between PLF-1 and sPLF sequences, and the location of the deleted fragment in the shorter one, would be in keeping with the occurrence of an alternative splicing process of the primary PLF transcripts in Ehrlich carcinoma cells. Linzer and Wilder observed dif-

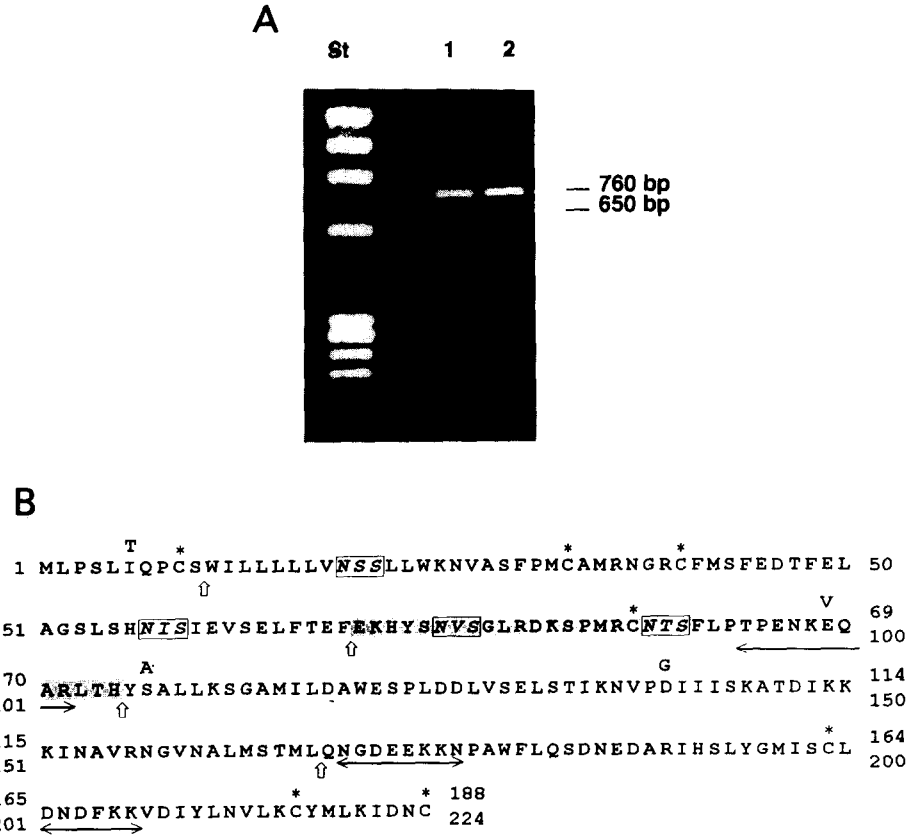

Fig. 4. Different RT/PCR amplification products obtained from Ehrlich cell samples. (A) Analysis by agarose electrophoresis and ethidium bromide staining of 10 μl/lane of RT/PCR products (22 cycles) from mRNA samples obtained from cells harvested at plateau-phase of growth in vivo (lane 1), and the same cells growing in vitro after 12 h of primary culture (lane 2). In both cases, all of the mRNA extracted from 1×10^6 cells was used for RT/PCR in a final volume of 100 μl. *Hae*III-digested φX-174 was used as standard (lane St). (B) Deduced amino acid sequences encoded by MRP/PLF mRNAs of Ehrlich cells. The fragment not encoded by sPLF is shadowed. Potential glycosylation sites are in boxes. Cys residues are marked with asterisks. Horizontal arrows underline the highest surface probability fragments. Vertical arrows indicate the exon junction sites in the *mrp/plf* mature transcripts [10]. The amino acid substitutions respect to PLF-1 found in two of the five sequenced clones are shown on the sequence at their respective positions; PLF-AII: Thr^6 (ACT), Val^{99} (GTA), Ala^{107} (GCA); and sPLF-A: Gly^{103} (GGT).

ferences in *mrp/plf* transcript processing between resting and serum-stimulated 3T3 fibroblasts [14]. More recently, Malyankar et al. [5] report that alterations in the processing pathway play a critical role in MPR/PLF transcript stability during cultured murine fibroblast immortalization. Nevertheless, the existence of shorter MRP/PLF mRNA variants has not been observed on mouse fibroblasts [5,14]. The occurrence of these different MRP/PLF-related open reading frames in Ehrlich carcinoma cells seem to be a growth-independent fact, since the same amplification pattern was obtained with cells in plateau-phase of growth and cells growing in culture for 12 h (Fig. 4a).

In conclusion, in this letter we communicate for the first time the occurrence of difference PLF-1-related mRNAs in Ehrlich carcinoma cells. In addition, the expression of these MRP/PLF mRNAs do not seem to be related to cell growth in this model. Repressors of MRP/PLF expression (i.e. glucocorticoids, TGF-β) [2] could also operate during lag and exponential phase of growth in vivo. In fact, tumor transplantation of Ehrlich cells is brought about using cells expressing MRP/PLF actively (day 10–day 12). Ehrlich carcinoma-bearing mice are well-characterized in terms of host–tumor interactions [17,18,25]. In addition, since Ehrlich cells proliferate in serum-free culture media, individual effects of hormones, mitogens and growth factors on the MRP/PLF expression could be clearly observed and correlated with the results obtained in vivo. Thus, Ehrlich carcinoma seem to be a good model for studying the physiological role and the control of the expression of these proteins in transformed cells.

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