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Instability of Endogenous MRP/Proliferin Transcripts in the Nucleus of Mouse Embryo Fibroblasts Contrasts With Their Stability When Produced During Transient Transfections

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Abstract The mitogen regulated protein/proliferin (MRP/PLF) gene is transcribed in primary mouse embryo fibroblasts (MEFs), but the pre-mRNA is not properly converted into a stable cytoplasmic mRNA and instead is rapidly degraded, apparently in the nucleus [Malyankar et al. (1994): Proc Natl Acad Sci USA 91:335–359]. In 3T3 cells derived from the MEFs by the standard 3T3 immortalization protocol, stable MRP/PLF mRNA is produced. We show here that the processing of intron sequences is similar in the two cell types and that some of the MRP/PLF transcripts are polyadenylated in the MEFs. We also document the production of stable MRP/PLF mRNA generated by transcription of various plasmid constructs containing different portions of the MRP/PLF3 gene after calcium phosphate-mediated transfection into the MEFs. We conclude that the inability of the MRP/PLF mRNA to accumulate in the MEFs is unlikely to result solely from a single localized sequence in the primary transcript (or the mRNA) that causes it to be subject to rapid breakdown; possibly export of the mRNA from the MEF nucleus is defective or some aspect of the transcriptional process marks the transcript for degradation. © 1996 Wiley-Liss, Inc.

Key words: post-transcriptional regulation, RNA stability, mRNA export, gene regulation, transient transfections, immortalization

Previous research has established that the mitogen-regulated protein/proliferin (MRP/PLF) gene is transcribed in primary mouse embryo fibroblasts (MEFs) but that the transcript is rapidly degraded [Edwards et al., 1987; Malyankar et al., 1994]. In 3T3 cells, derived from the mortal MEFs according to the "immortalization" regimen described by Todaro and Green [1963], the MRP/PLF transcript is in contrast processed into a reasonably stable and functional mRNA [Parfett et al., 1985; Linzer et al., 1985]. Why a stable mRNA is not produced in the MEFs is of considerable interest since this would appear to represent a novel level of gene regulation that is not understood and may be important in conferring the mortal phenotype on normal cells.

MRP/PLF encodes a secreted protein that belongs to the growth hormone/prolactin family and is involved in fetal–maternal interactions in mid pregnancy in the mouse [Nilsen-Hamilton et al., 1988; Lee et al., 1988; Connor et al., 1989]. It can also promote endothelial cell migration and angiogenesis [Jackson et al., 1994]. Receptors have been identified in preparations from mid-gestation mouse uteri and shown to convey a proliferative signal to the cells upon stimulation with MRP/PLF [Nelson et al., 1995]. In the mouse MRP/PLF mRNA is found at significant levels only in midgestation placenta; whether the gene is transcribed but not expressed in other tissues is not known.

The relationship between the acquisition of the immortal phenotype and the ability to generate MRP/PLF mRNA is unclear. However, given the ability of this protein to regulate transcription in certain cell types [Muscat et al., 1991], it

Abbreviations used: CMV, cytomegalovirus; HIV-1, human immunodeficiency virus type 1; MEF, mouse embryo fibroblast; MRP, mitogen regulated protein; PAS, polyadenylation signal; PLF, proliferin; RT-PCR, reverse transcription-polymerase chain reaction; VIM, vimentin.

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is possible that MRP/PLF does play a role in immortalizing 3T3 cells. MRP/PLF expression is not a general requirement for immortalization of mouse cells since a number of immortal lines do not express it [Parfett and Denhardt, 1989]. Also, expression does not seem to be required for the proliferation of Ehrlich carcinoma cells [Gil-Torregrosa et al., 1994].

In this research we have used an intron probe and RT-PCR analysis to study processing of the MRP/PLF transcript. We find that the endogenous MRP/PLF transcript appears to be processed in MEFs as it is in 3T3 cells, except that no stable mRNA is produced. We have expressed all but some 350 nt of the primary transcript in MEFs using a calcium phosphate-mediated transfection procedure and, paradoxically, we find that stable mRNA can be produced from the transfected DNA, suggesting that the endogenous MRP/PLF transcript is degraded in MEFs by a process that does not depend only on sequences in the transcript. We propose that this paradox reflects an aspect of mRNA translocation that is dependent on chromatin structure and nuclear organization.

MATERIALS AND METHODS

Preparation of MEFs and Cell Culture

Primary MEFs were prepared from 14- to 16-day-old mouse embryo bodies. Cells were cultured in Dulbecco's modified essential medium (DMEM) with 10% calf serum plus penicillin and streptomycin at an initial plating density of 1.7×10^4 cells/cm^2 [Todaro and Green, 1963]. After two passages, batches of the primary MEFs were frozen in DMEM containing 5% dimethylsulfoxide (DMSO) and 20% calf serum and stored in liquid nitrogen. A number of 3T3 cell lines were established by passaging the cells through the crisis period, which was monitored by the increase in cell number with each passage. These cell lines were then analyzed for MRP/PLF expression and used for transfection and other studies [Denhardt et al., 1991; Rittling and Denhardt, 1992]. Subconfluent cells were refed with DMEM containing 10% calf serum 12 h prior to harvest for the studies reported here, because it was previously shown that levels of MRP/PLF message were maximal after this time of stimulation [Parfett et al., 1985].

RNA Isolation and Northern Analysis

Total RNA was isolated by the guanidinium thiocyanate–phenol–chloroform method [Chomczynski and Sacchi, 1987], using either the TriReagent protocol or available laboratory reagents. Poly(A)$^+$ RNA was prepared from total RNA using the PolyATtract System (Promega, Madison, WI), which employs oligo(dT) linked to magnetic particles.

Nuclear RNA was prepared by first isolating the nuclei as described by Ausubel et al. [1989]. Briefly, cells were washed with ice-cold phosphate-buffered saline, removed from the surface by scraping, and centrifuged at 500*g* for 5 min at 4°C. The cells were lysed for 5 min on ice using 4 ml of NP-40 lysis buffer (10 mM Tris–HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40) for every 10^7 cells. Nuclei were separated by centrifugation at 500*g* for 5 min at 4°C. The supernatant was used for isolation of cytoplasmic RNA after proteinase K treatment and extraction with phenol–chloroform–isoamyl alcohol. The nuclear pellet was subjected to a second round of lysis buffer treatment and the supernatant discarded. The nuclei were lysed and RNA purified using guanidinium thiocyanate–phenol–chloroform [Chomczynski and Sacchi, 1987].

Northern analysis of RNA was carried out by fractionating the indicated amount (usually 10 μg) of RNA on a 1% agarose gel containing formaldehyde [Ausubel et al., 1989]. Upon completion of the electrophoresis, the gels were examined by ethidium bromide staining, and the RNA was transferred onto Genescreen Plus nylon membranes by capillary blotting and crosslinked to the filter matrix by ultraviolet (UV) radiation. Filters were prehybridized in a solution containing 50% deionized formamide, 10% dextran sulfate, 1% sodium dodecyl sulfate (SDS), 1 M NaCl, 100 μg/ml denatured salmon sperm DNA for 1 h at 42°C. DNA probes were labled using the random prime kit from Pharmacia (Piscataway, NJ) and [α^{32}P]dCTP (3000 Ci/mmol) and used at a concentration of $2–5 \times 10^5$ cpm/ml in the hybridization mix. Blots were washed in 1× SSC, 0.1% SDS at 65°C. Probes were stripped from the blot by boiling the filter in a solution of 0.1× SSC and 1% SDS for 30 min. SSC (standard saline citrate) is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.

RT-PCR Analysis of Transcripts

Reverse transcriptase reactions were carried out using M-MLV reverse transcriptase (Gibco-BRL, Gaithersburg, MD). RNA was treated with RNase-free DNase to prevent the amplification of DNA fragments. Random hexamer primers

served to prime synthesis of the cDNA strand by reverse transcriptase. The reaction was carried out at 37°C for 60 min in a Perkin Elmer Cetus Thermal Cycler following which the enzyme was denatured by heating the tubes at 95°C for 10 min. The efficiency of the cDNA reaction for the different cell lines was calculated by performing a parallel reaction with a trace amount of [α^{32}P]dCTP (3,000 Ci/mmol) and determining the amount of isotope incorporated. The tubes were cooled and the reaction was divided into 5 equal aliquots of 5 μl, each of which was then used in a PCR reaction (see Fig. 3). The concentrations of the primers and the dNTPs were 0.2 μM and 200 μM, respectively. The $MgCl_2$ concentration was 1.5 mM and the final volume was 100 μl. PCR primers used in the analyses were chosen using the PCRPRIM program [Lowe et al., 1990].

RNase Protection Assays

MEF and 3T3 cells were transfected in parallel, by calcium phosphate coprecipitation [Graham et al., 1980], with 10 μg of pCMVMRP, pCMVMRP3U, or pCMVGΔE together with 10 μg pCMVVIM as an internal control; 72 h after transfection the cells were harvested and total RNA purified as above. The CMV portion of the plasmid is the CMV promoter obtained from pCMVCAT [Foecking and Hofstetter, 1986]. pCMVVIM expresses the cDNA of human vimentin [Sommers et al., 1989]. Structures of these plasmids and the pPEHI plasmid are shown in Figures 4 and 5; details of their construction may be found in [Malyankar, 1994]. The *Nco*I–*Mae*I fragment of pCMVMRP containing 319 bases of CMV sequence and 383 bases of MRP/PLF cDNA sequence was cloned into pGEM3ZF+; transcription of this plasmid with SP6 polymerase in the presence of [α^{32}P]UTP or [α^{32}P]CTP yielded the ^{32}P-labeled antisense CMV/MRP probe used in the RNA protection analyses in Figures 4 and 5. The genomic 3′ antisense probe used in Figure 6 was synthesized using T7 polymerase and pGMRP2 [Connor, 1989] cleaved with *Pvu*II as the template.

RNA probes were labeled to a high specific activity using [α^{32}P]CTP (800 Ci/mmol) [Melton et al., 1984]. Production of full-length RNA probes was favored by using a slightly higher concentration of unlabeled nucleotides (0.75 mM each) and by incubating the reactions at 30°C instead of 37°C. Unincorporated nucleotides were removed by ethanol precipitation. The RNA samples were hybridized with 5×10^5 cpm of ^{32}P-labeled probe per reaction using 10 or 20 μg of RNA at 45°C (39°C for the 3′ antisense probe) for 16 h in a buffer consisting of 80% formamide, 8 mM piperazine-N,N′-bis[2-ethanesulfonic acid] (PIPES) pH 6.4, 80 mM NaCl, and 0.2 mM EDTA. The annealed samples were then incubated with 100 units of RNase T1 and 6 μg of RNase A in a buffer containing 10 mM Tris–HCl, pH 7.5, 300 mM NaCl, and 5 mM EDTA. The resulting products were electrophoresed on a 6% urea–polyacrylamide gel at 60 watts constant power. Gels were dried and autoradiographed.

RESULTS

Processing of the MRP/PLF Transcript in MEF and 3T3 Cells

Using PCR primers that spanned exon–intron boundaries, Malyankar et al. [1994] established that the abundance of the primary MRP/PLF transcript in MEF cells was about one-half that in 3T3 cells. In this study we have characterized further some of the properties of the transcripts produced in the MEFs. Figures 1 and 2 show results from experiments using the cDNA probe and an intron 2 probe to compare the processing of the primary transcript in MEFs with its processing in 3T3 cells. Unlike some of the MRP/PLF intron probes, this intron 2 probe does not contain any repetitive sequences, as judged from its hybridization pattern to southern blots of genomic DNA (D. Oscislawski and S. Rittling, data not shown). We therefore believe that it is specific for the MRP/PLF intron 2 sequence.

Figure 1 shows a northern blot of the poly(A)$^+$ and poly(A)$^-$ fractions (1 μg and 10 μg, respectively, were electrophoresed) isolated from the total RNA (nuclear + cytoplasmic) of 3T3 and MEF cells probed with the cDNA and with the intron 2 probe. Abundant processed MRP/PLF mRNA was seen in the polyadenylated fraction from 3T3 cells, but not from MEFs. Unprocessed and incompletely processed transcripts were detected mostly in the poly(A)$^-$ fraction from both cell types, with the abundance and size distribution being larger in 3T3 cells than in MEFs.

The stability of the MRP/PLF transcripts was assessed using actinomycin D to inhibit synthesis of the RNA. RNA was extracted from preparations of nuclei after different periods of exposure of the cells to actinomycin D and analyzed for the abundance of the MRP/PLF transcript using a cDNA probe (Fig. 2A) and an intron 2

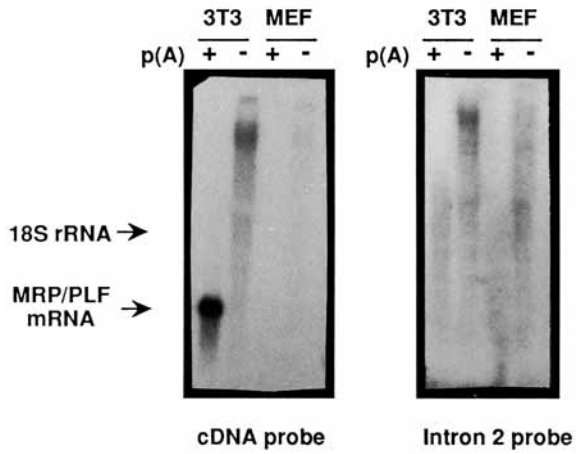

Fig. 1. Analysis of poly(A)$^+$ and poly(A)$^-$ fractions from total cellular RNA of 3T3 and MEF cells. One μg of poly(A)$^+$ RNA and 10 μg of poly(A)$^-$ RNA from 3T3 and MEF cells were used to produce the Northern blot. The blot was first probed with a cDNA probe for MRP/PLF mRNA, then stripped and probed with an intron 2 probe (a 2-kb *Pst*I–*Bam*HI fragment [Connor et al., 1989]). Analysis of the blots with an actin probe and for 18S RNA confirmed the quality and equality of loading of RNA in the poly(A)$^+$ and poly(A)$^-$ fractions, respectively.

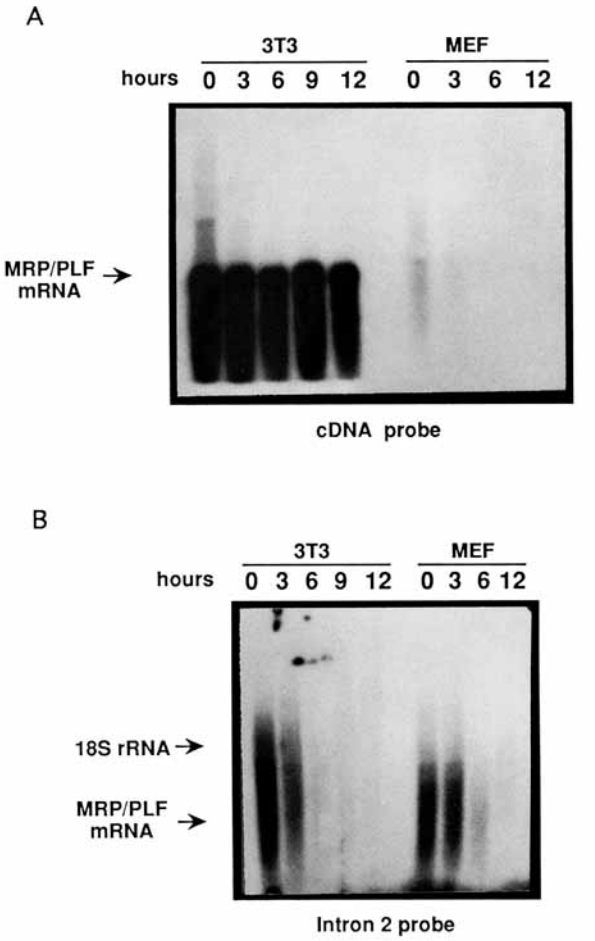

Fig. 2. Determination of the stability of the MRP/PLF transcript. Cells were harvested at the indicated times (hours) after the addition of actinomycin D (5 μg/ml) to subconfluent 3T3 and MEF cells. Ten μg of nuclear RNA, isolated as described under Materials and Methods, was electrophoresed and blotted. **A:** Blot probed with MRP/PLF cDNA. The MRP/PLF signal was largely in the position of the intact mRNA but, because of the overexposure of the film, this is not evident in the picture. **B:** The blot was then stripped and reprobed with the 2-kb *Bam*HI–*Pst*I intron 2 probe. Analysis for 18S rRNA was performed to confirm the integrity of the RNA and the equality of loading.

probe (Fig. 2B). Figure 2A shows that an abundance of stable MRP/PLF transcripts could detected with the cDNA probe in 3T3 nuclei (likely contaminated with cytoplasmic RNA); the half-life of the MRP/PLF mRNA in these preparations was estimated from a lighter exposure to be somewhat over 12 h. In the MEFs, MRP/PLF mRNA was only faintly detected, in this overexposed film, and as expected had a much shorter half-life. As in Figure 1, there was much less disparity in the amount of MRP/PLF RNA detected with the intron 2 probe than with the cDNA probe in the two cell types, and again its average size in the MEFs was smaller (Fig. 2B). This is consistent with the diminished stability of the MRP/PLF transcript in these cells. The process of isolating nuclei permits some additional processing (and degradation?) of the MRP/PLF RNA that does not occur when the RNA is extracted directly from intact cells, likely accounting for the smearing and smaller size of the MRP/PLF RNA compared to Figure 1. The MRP/PLF RNA detected in the nuclear RNA with the intron 2 probe was largely gone after 6 h in the presence of actinomycin D in both MEF and 3T3 cells. Since the intron 2 probe likely hybridizes to a mixture of species, including partially processed transcripts and the excised intron itself, the determination of a half-life is not very useful in this case.

A detailed examination of the polyadenylated and nonpolyadenylated RNA present in MEFs and 3T3s using various PCR primer pairs to detect different portions of the MRP/PLF transcript is shown in Figure 3. The exon–intron primers from the 5′ region spanning the exon 1–intron 1 boundary (lanes 1–4) and the 3′ region spanning the intron 4–exon 5 boundary (lanes 5–8) both amplified a PCR fragment of the expected size (approximately 300 bp for the former and 400 bp for the latter) in both cell types. The somewhat less intense signal in the MEF lanes as compared to the 3T3 lanes is in

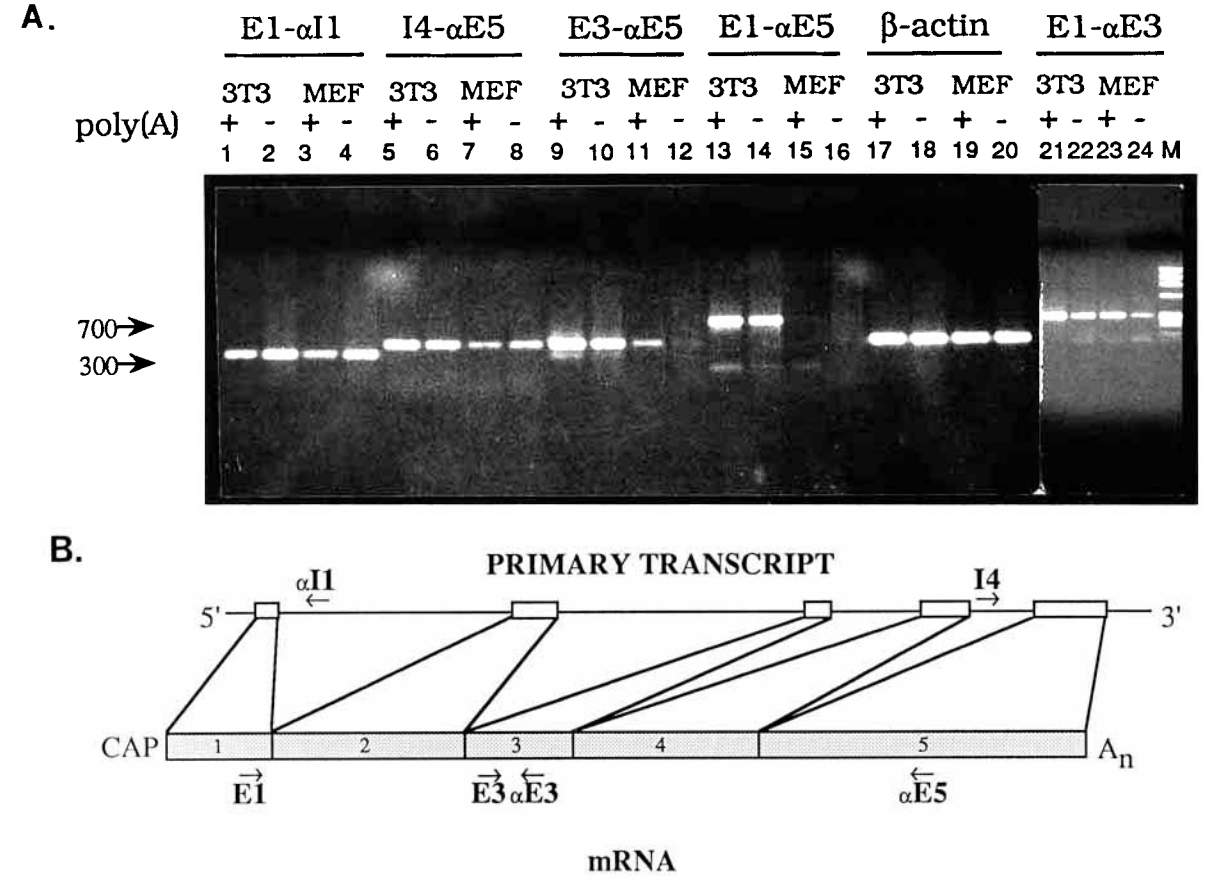

Fig. 3. PCR amplification of sequences in poly(A)$^+$ and poly(A)$^-$ RNA from MEF and 3T3 cells. Odd numbers, RT-PCR carried out using poly(A)$^+$ RNA; even numbers, RT-PCR carried out using poly(A)$^-$ RNA. The gel for *lanes 21–24* and M (markers) was run differently from the gel for *lanes 1–20*. Lanes 1, 2, 5, 6, 9, 10, 13, 14, 17, 18, 21, 22, RT-PCR reactions of 3T3 RNA; lanes 3, 4, 7, 8, 11, 12, 15, 16, 19, 20, 23, 24, RT-PCR reactions of MEF RNA; lanes 1–4, PCR employing exon 1 and antisense (α) intron 1 primers; lanes 5–8, PCR employing intron 4 and antisense (α) exon 5 primers; lanes 9–12, PCR employing exon 3 and antisense (α) exon 5 primers; lanes 13–16, PCR employing exon 1 and antisense (α) exon 5 primers; lanes 17–20, PCR employing β-actin sense and antisense primers; lanes 21–24, PCR employing exon 1 and antisense (α) exon 3 primers. The primers used were E1, 5'CCCTTCTTCGATTCAACCATG3'; αI1, 5'AGACACTGCTGCATACTCTAGG3'; I4, 5'ACAACAAACCCATCTCAGG3'; αE5, 5'CATGTAACACTTCAGGACG3'; E3, 5'TATTCTAACGTGTCTGGGC3'; αE3, 5'CCTTGTTTTCTGGAGTTGG3'; 5' β-actin, 5'GCCAGGTCATCACTATTGG3'; 3' β-actin: 5'AGTAACAGTCCGCCTAGAAGC3'.

harmony with the earlier conclusion that the primary transcript in the MEFs can be detected at roughly one-half the abundance observed in 3T3 cells. In all the RT-PCR reactions, preliminary experiments were carried out without reverse transcriptase to check for DNA contamination (none was detected, data not shown).

We used exon 1 "sense" and exon 3 "antisense" primers (E1-αE3, 290 bp, lanes 21–24), and exon 3 "sense" and exon 5 "antisense" primers (E3-αE5, 440 bp, lanes 9–12) to examine splicing of introns 1 and 2, and introns 3 and 4, respectively. The evident signal in the MEF lanes, including the poly(A)$^+$ fractions, establishes that these introns can be spliced out. Larger DNA species corresponding to fragments that contain introns are not seen because they are discriminated against during the amplification process; thus this assay is specific for spliced transcripts. (Note that the DNA in lanes 21–24 and M was electrophoresed in a different gel than the DNA in lanes 1–20, and therefore the sizes cannot be directly compared.) The rapidly migrating species seen in some reactions (lanes 13–15, 21–24) are likely PCR artifacts. Actin mRNA was used as a control for RNA integrity in these experiments (lanes 17–20).

The fragment amplified by the E1-αE5 primers represents the fully spliced mRNA. It migrated at about 700 bp and was prominent in the 3T3 RNA (lanes 13, 14). Although in this experiment a faint signal was detected in the MEF

poly(A)$^+$ preparation as well (lane 15), this was not a reproducible observation. One explanation for our occasional detection of very low levels of MRP/PLF mRNA in the MEFs may be that a few cells in the MEF population have acquired the ability to produce stable MRP/PLF mRNA. The appearance of strong bands in lanes 11 and 23 indicates polyadenylation of incompletely spliced MRP/PLF transcripts. These lanes represent poly(A)$^+$ MEF mRNA, which contains little or no fully spliced mRNA (lane 15). Taken together, these data establish that in MEFs splicing of all the introns can occur and that the partially spliced transcripts are polyadenylated.

Production of Stable MRP/PLF3 mRNA from Plasmids Transfected into MEFs

We have shown by RNase protection that after transfection of a plasmid containing the MRP/PLF cDNA under the control of the CMV promoter (pCMVMRP) into MEF and 3T3 cells the MRP/PLF message could accumulate to comparable levels in both cell types after correction for the transfection efficiencies [Malyankar et al., 1994]. The experiments described in Figure 4 employed a similar RNase protection strategy using plasmid constructs that contained different portions of the MRP/PLF3 gene. As diagrammed in Figure 4C, the endogenous MRP/PLF message protects a 383-nt fragment, whereas RNA transcribed from the transfected MRP/PLF construct protects a 479-nt fragment that includes MRP/PLF exons 1–3 plus part of exon 4 and some 5′ untranslated sequence from the CMV promoter. As controls for transfection efficiency in these experiments, an analogous construct encoding the stable vimentin mRNA was cotransfected and the level of the ~96-nt CMV portion of the CMV-vimentin transcript assayed simultaneously (data not shown; see Figure 5 in Malyankar et al. [1994]).

Figure 4A shows that correctly spliced mRNA (479 nt) can be detected when the MRP/PLF3 gene in the 13.8-kbp pCMVGΔE plasmid is transfected into the MEF and 3T3 cells. Quantitation of the RNase protection results and correction for transfection efficiencies suggested that the processing of the transcript was similar, giving rise to the same protected fragments, in both cell types. The smaller fragments are of a size consistent with their representing splicing intermediates or individual exons; exons 1–5 are, respectively, 100, 165, 120, 200, and 290 nt. The relative (to CMV VIM) abundance of the MRP/PLF transcripts was 4.2 and 1.5 for CMV MRP and CMVGΔE in 3T3 cells and 29.2 and 1.3 for CMV MRP and CMVGΔE in MEFs, respectively. We believe that the result for CMV MRP in MEFs is anomalously high for unknown reasons; in other experiments pCMVMRP was expressed at similar levels in 3T3 and MEF cells (Fig. 4B, Malyankar et al., 1994; Malyankar and Rittling, unpublished data). We have therefore concluded that the introns do not usually affect the stability of the transcript in the transfected cells.

Missing from the GΔE construct are 55 nt from the extreme 5′ end and 300 nt internal to intron 3 that corresponds to an *Eco*RI fragment (hence ΔE) that has been refractory to cloning. Other differences from the endogenous gene include the absence of DNA sequences upstream of the transcription start site (the promoter) and downstream beyond about 500 nt past the polyadenylation site.

To address the question of whether sequences downstream of the polyadenylation site might be determining the instability of the MRP/PLF transcript in MEFs, the plasmid pCMVMRP3U, diagrammed in Figure 4B, was constructed. This plasmid contains 3.5 kbp of DNA downstream of the polyadenylation site joined to the 3′ end of the MRP/PLF cDNA. When this construct was transfected into 3T3 and MEF cells, sufficient MRP/PLF RNA was detected at 479 nt in both transfected cell types to suggest that this downstream sequence did not destabilize the MRP/PLF message under these conditions.

In both experiments (Fig. 4A,B), endogenous MRP/PLF mRNA (at 383 nt) was evident in 3T3

Fig. 4. RNase protection assay of RNA transcribed from transfected pCMVGΔE and pCMVMRP3U. **A:** Map of pCMVGΔE and an autoradiograph of a denaturing gel analysis of the protected RNA fragments from cells transfected with pCMVMRP and pCMVGΔE. Δ, Approximate position of a 300-bp *Eco*RI fragment that is missing from intron 4. Protected RNA from the pCMVMRP plasmid and from the pCMVGΔE plasmid (fully spliced) is 479 nt long, while the endogenous transcript is 383 nt long. A number of the bands seen in the pCMVGΔE lanes in both the MEF and 3T3 lanes correspond to one or a combination of exons arising from partially spliced transcripts. **B:** Map of pCMVMRP3U and an autoradiograph of the protected fragments. Protected bands from co-transfected CMVVIM were used to normalize for transfection efficiency and loading differences (not shown). The pCMVMRP lanes are included to illustrate expression of the cDNA driven by the CMV promoter. **C:** Sketch showing the sizes of the protected bands and the probe used. The 3′ end of the probe is at a MaeI site in about the middle of exon 4.

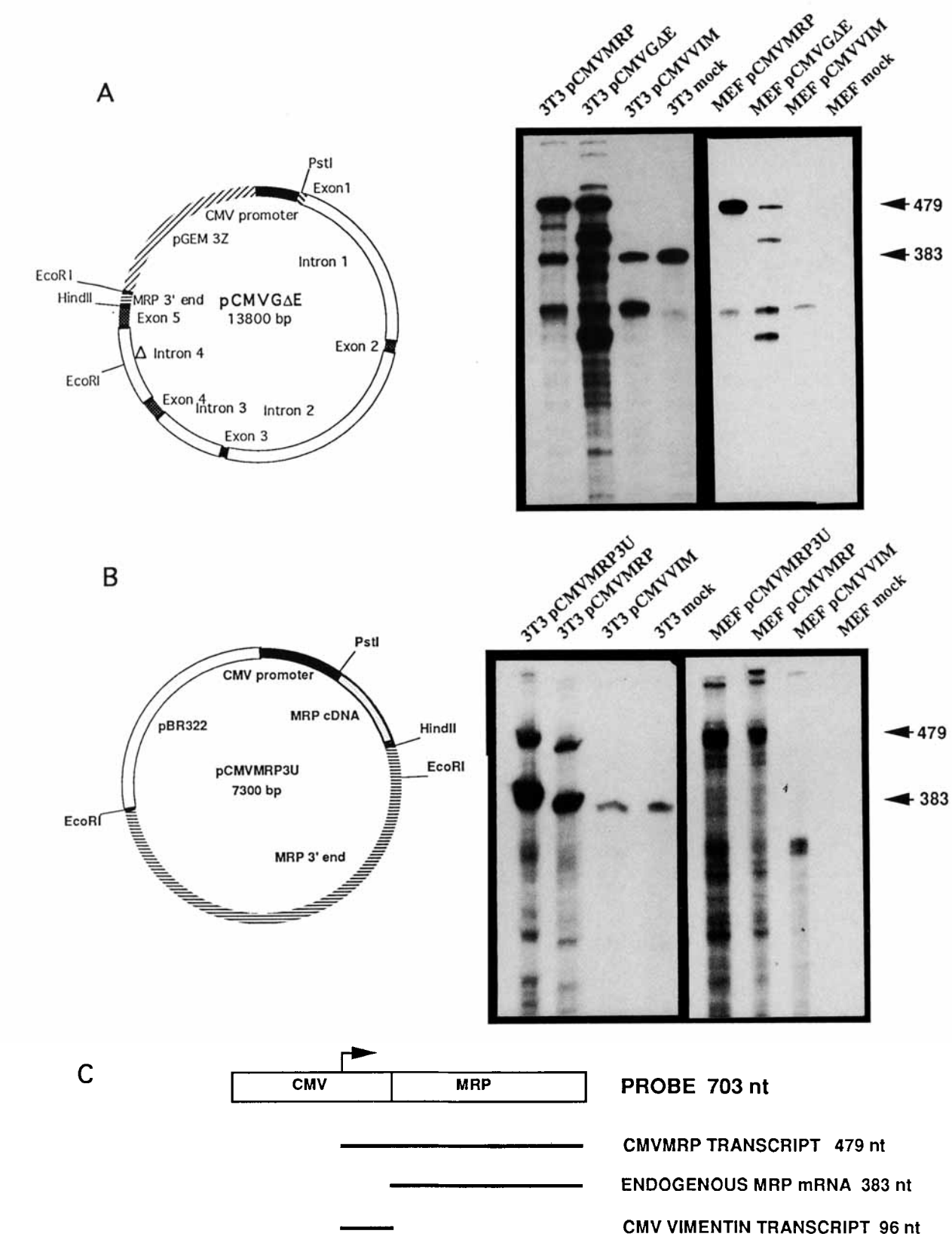
A
PstI
Exon1
CMV promoter
pGEM 3Z
Intron 1
EcoRI
HindII
MRP 3' end
Exon 5
pCMVGΔE
13800 bp
Exon 2
Δ Intron 4
EcoRI
Exon 4
Intron 3
Intron 2
Exon 3
3T3 pCMVMRP
3T3 pCMVGΔE
3T3 pCMVVIM
3T3 mock
MEF pCMVMRP
MEF pCMVGΔE
MEF pCMVVIM
MEF mock
479
383
B
PstI
CMV promoter
MRP cDNA
pBR322
HindII
EcoRI
pCMVMRP3U
7300 bp
EcoRI
MRP 3' end
3T3 pCMVMRP3U
3T3 pCMVMRP
3T3 pCMVVIM
3T3 mock
MEF pCMVMRP3U
MEF pCMVMRP
MEF pCMVVIM
MEF mock
479
383
C
CMV
MRP
PROBE 703 nt
CMVMRP TRANSCRIPT 479 nt
ENDOGENOUS MRP mRNA 383 nt
CMV VIMENTIN TRANSCRIPT 96 nt

Figure 4

cells, but not in MEFs. When the amount of the CMV-generated MRP/PLF transcript (at 479 nt) in both cell lines was normalized for transfection efficiency (by densitometric comparison to the cotransfected pCMVVIM transcript), the calculated levels of MRP/PLF transcript in the transfected 3T3 and MEF cells appeared comparable.

To address the question of whether it was the promoter that determined the instability of the MRP/PLF transcript, we assessed expression of the MRP/PLF cDNA driven by a ~1.1-kb portion [−1101 to +64; Connor et al., 1989] of the MRP/PLF3 promoter in pPEHI (Fig. 5A). Subsequent to transient transfection of MEF and 3T3 cells with this plasmid, the cellular RNA was extracted and analyzed by RNase protection using the probe described in Figure 4C. As documented in Figure 5B, the protected band at 383 nt indicates that stable MRP/PLF mRNA can accumulate in the MEFs transfected with the PEHI plasmid. (The extent of expression in 3T3 cells cannot be assessed since the 383-nt MRP/PLF transcript encoded in the pPEHI transcript is indistinguishable from the endogenous 3T3 transcript.) These results suggest that this part of the MRP/PLF3 gene does not determine the stability of the transcript, consistent with our previous finding that the MRP/PLF3 promoter directs expression equivalently in MEF and 3T3 cells [Malyankar et al., 1994].

Processing of the 3′ End of the MRP/PLF3 Transcript

Since some of the signals responsible for determining the sensitivity of specific mRNAs to degradative pathways are located at the 3′ end of the message [Sachs, 1993], the importance of the 3′ end of the MRP/PLF gene in conferring transcript instability was investigated in MEF and 3T3 cells using a probe that included most of the last exon and extended 275 nt downstream of the end of the cDNA. The RNase protection analysis of the total cellular RNA illustrated in Figure 6 revealed a 195-nt fragment corresponding to the expected cleavage site approximately 10 nt downstream of an AAUAAA polyadenylation signal (PAS); it was detected in 3T3 cells, and to a lesser extent in

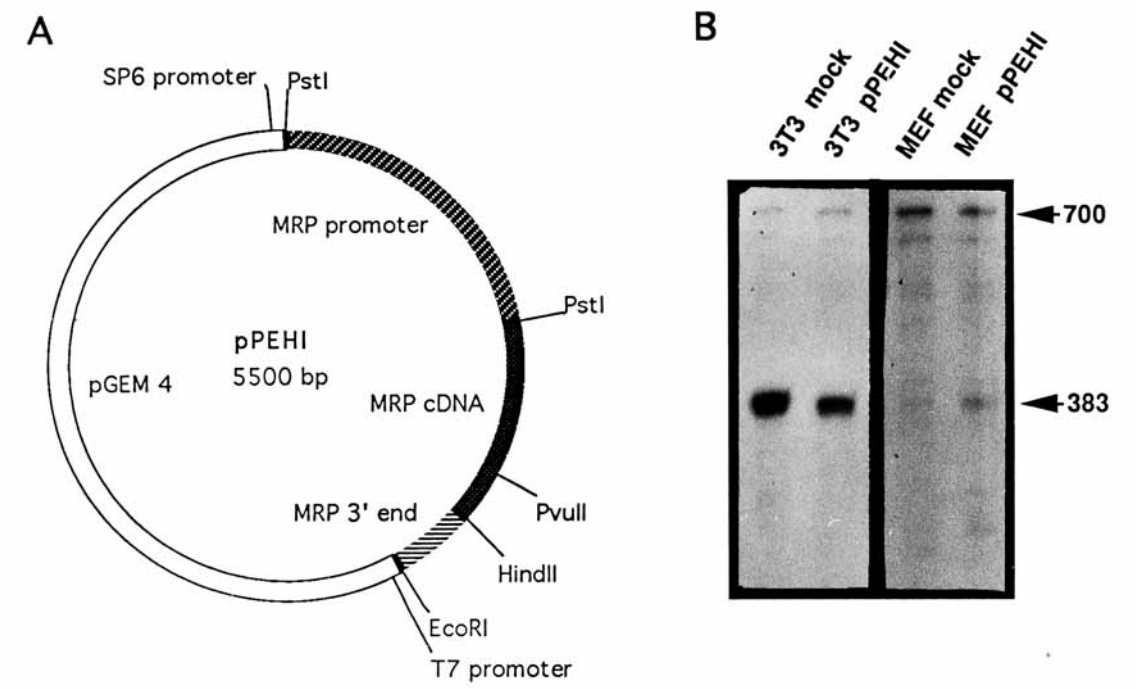

Fig. 5. MRP/PLF cDNA driven by an MRP/PLF promoter is expressed in MEF cells. **A:** Map of the pPEHI construct. Transcription of the MRP/PLF cDNA is under the control of the 1.1-kb *Eco*RI–*Pst*I promoter segment; downstream of the cDNA is the 0.46-kb 3′ *Hin*dII–*Eco*RI segment [Connor et al., 1989; Malyankar, 1994]. **B:** RNase protection assay of the RNA present in the pPEHI-transfected cells using the probe diagrammed in Fig. 4C. The lack of a 383-nt fragment in the MEF mock lane is the control for the signal observed in the MEF PEHI lane. In this experiment, pMRPLUC was transfected into identical plates and the amount of luciferase expressed was used to normalize transfection efficiencies [Malyankar et al., 1994].

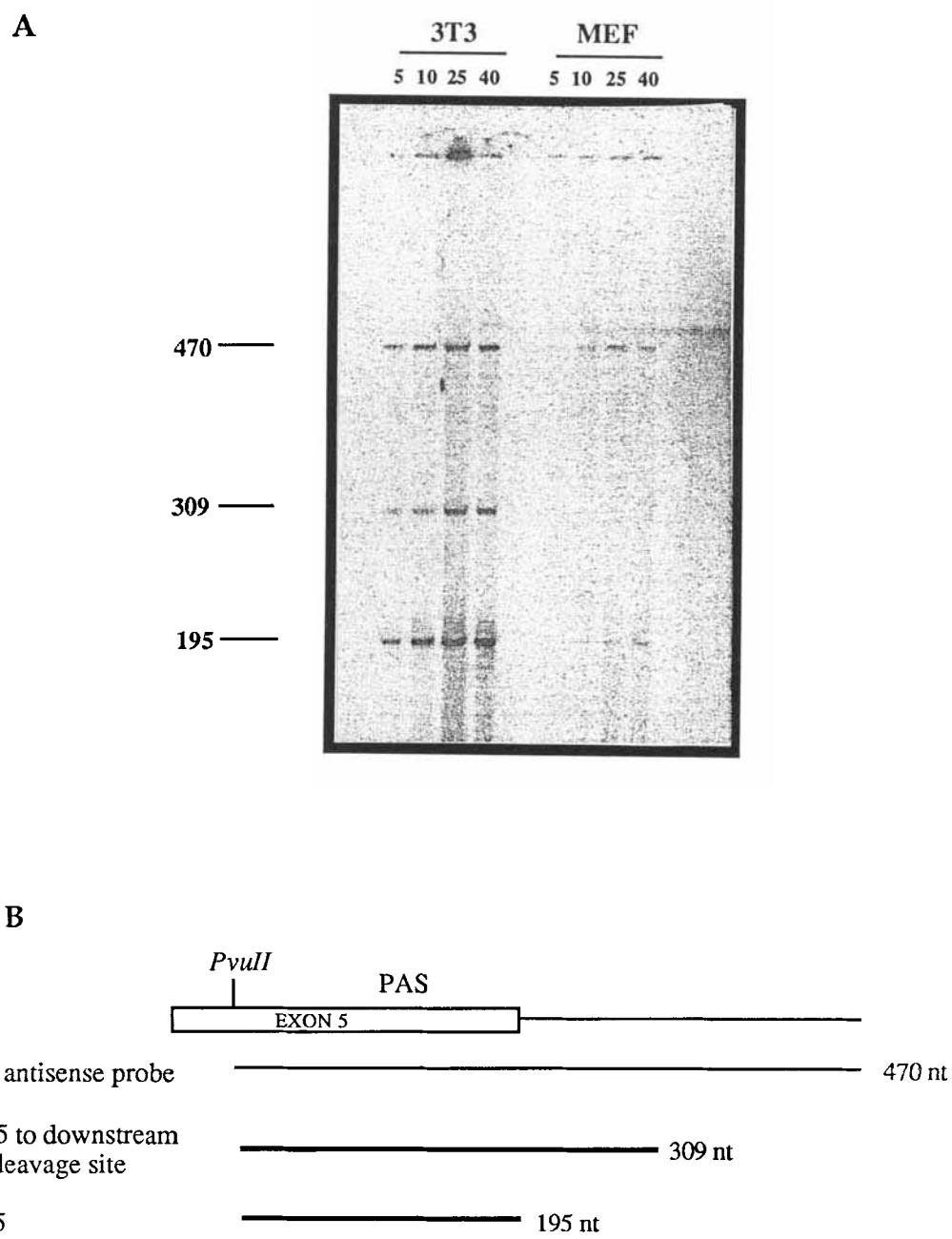

Fig. 6. Normal termination at the 3′ end of the MRP/PLF gene in MEFs. **A:** RNase protection assay of the total RNA (5–40 μg) from subconfluent 3T3 and MEF cells. **B:** The probe was antisense RNA extending from the *PvuII* site in exon 5 to the end of the available 3′ untranslated region (approximately 470 nt). PAS, Normal polyadenylation signal that gives rise to the 195-nt E5 fragment.

the MEFs. This is evidence that the entire MRP/PLF gene can indeed be transcribed and can terminate normally in the MEFs. In addition, and unexpectedly, a 309-nt band was detected in 3T3 cells, but apparently not (at least to the same extent) in the MEFs. Inspection of the nucleotide sequence for possible polyadenylation signals revealed a CAUAAA sequence in about the correct location for generating the 309-nt band. Interestingly, the 309-nt species observed in Figure 6 was not detected in an analysis of the polyadenylated fraction, suggesting perhaps that the cleavage mediated (we presume) by the CAUAAA sequence gives rise to a product that cannot be polyadenylated [Malyankar, 1994].

DISCUSSION

Search for a Destabilizing Element in the MRP/PLF Transcript

The experiments reported above have extended our understanding of the processing of the MRP/PLF transcript in the MEFs. As assessed by northern blotting with both the cDNA and intron 2 probes, the MRP/PLF transcript was present in nonpolyadenylated RNA from both MEF and 3T3 cells, with the average size and abundance being greater in the 3T3 cells. MRP/PLF transcripts could also be detected in the poly(A)$^+$ RNA from both cell types. Although the cDNA probe revealed mature mRNA at substantial levels only in the 3T3 cells, the intron 2 probe detected comparable amounts of hybridizing RNA in both cell types. The presence of polyadenylated RNA containing intron 2 sequences (Fig. 1) suggests that polyadenylation of the transcript can precede removal of intron 2; this is consistent with the results of the RT-PCR analyses (Fig. 3) revealing polyadenylation of partially spliced transcripts.

The stability of the transcripts was assessed using actinomycin D to inhibit RNA synthesis. The cDNA probe revealed the presence of a moderately stable MRP/PLF mRNA in 3T3 cells, but not in MEFs. The intron 2 probe revealed that sequences complementary to intron 2 were present at similar levels in both cell types with roughly comparable half-lives. When protein synthesis was inhibited by cycloheximide, a small amount of mature MRP/PLF mRNA was detected in MEFs in one experiment [Malyankar, 1994], but this was not a reproducible observation, suggesting that the process responsible for the degradation of the MRP/PLF transcript does not depend on an unstable protein.

We attempted to reproduce the instability of MRP/PLF RNA in MEFs by transient transfection with a plasmid using the CMV promoter to drive transcription of a genomic construct containing all the introns (pCMVGΔE). Previous studies had established that the processed transcript generated from a cDNA clone driven by the CMV promoter was stable in MEFs as well as 3T3 cells [Malyankar et al., 1994]. Results described above show that inclusion of essentially all of the intron sequences did not prevent the production of stable mRNA in transfected MEFs. The 300-nt missing *Eco*RI piece in intron 4 is unlikely to be solely responsible for the degradation of the transcript because if it were, one might expect that once intron 4 were excised (Fig. 3), the resulting transcript would be stable. The 55-nt segment missing from the 5′ end of the GΔE sequence is also not responsible for the instability, since it is present both in transcripts generated by the pPEHI construct (Fig. 5) and in the MRP/PLF3-luciferase construct studied by Malyankar et al. [1994].

It could be that the CMV promoter, a relatively strong promoter, expressed such a high level of the MRP/PLF transcript that the mechanism responsible for the targeted elimination of the MRP/PLF transcript in MEFs was overwhelmed. Alternatively, transfection of the MEFs using calcium phosphate could inhibit the destabilizing mechanism. Arguing against these possibilities is the observation that in transfected MEF cells production of stable MRP/PLF mRNA from the endogenous gene was never detected (Fig. 4A,B; also [Malyankar et al., 1994]). We cannot exclude the possibility that the introduced plasmid DNA, which is presumably not configured as mature chromatin, is not subject to the same regulatory controls as the endogenous gene [cf. Pennie et al., 1995]. Urlaub et al. [1989] noted this phenomenon and hypothesized that the position of a gene within the nucleus may determine aspects of its regulation. Alternatively, the secondary structure of the MRP/PLF RNA transcribed from the plasmids could differ from the secondary structure of the RNA generated from the endogenous genes, and this could account for the different stabilities.

To investigate whether it was necessary for transcription to be initiated from the MRP/PLF promoter itself in order for the destabilization mechanism to function, a plasmid with the MRP/PLF promoter driving expression of an MRP/PLF cDNA was constructed. MEF cells transfected with this plasmid (pPEHI) produced stable MRP/PLF mRNA, indicating that the promoter (at least this portion of it) was not responsible for the destabilization. (Our attempts to construct a plasmid with the MRP/PLF promoter driving transcription of the entire gene have not succeeded.) The question whether sequences downstream of the 3′ polyadenylation site were somehow responsible for destabilization of the transcript was studied by cloning an additional 3.5 kbp of DNA downstream from the normal termination site into pCMVVMRP and transfecting the resulting plasmid (pCMVMRP3U) into MEF and 3T3 cells. This construct expressed MRP/PLF to the same level as pCMVMRP, indi-

cating that these additional 3′ end sequences were not responsible for the destabilization.

Future research into this problem could include (1) a study using different transfection protocols with an extended segment of the MRP/PLF promoter together with the entire MRP/PLF gene to determine whether assembly of the transcriptional complex in the presence of downstream sequence elements is necessary to activate the regulatory mechanism; (2) a study of MEFs *stably* transfected with a marked MRP/PLF gene incorporated into the cellular chromatin and derived from transgenic mice to ascertain whether location in a chromosome is important; and (3) a study of the export of the MRP/PLF mRNA from the nucleus.

Other Examples of Intranuclear Regulation of the Stability of a Transcript

A number of publications (in addition to several noted earlier by Malyankar et al., 1994) describe various circumstances in which seemingly normal nuclear transcripts are degraded. These reports of intranuclear degradation of transcripts are clearly distinct from the destabilization of cytoplasmic mRNAs by UUAUUUA (U/A) (U/A) sequences located in the 3′ untranslated region of the mRNA [Lagnado et al., 1994].

Research on a variety of genes, for example, that by Urlaub et al. [1989] on the DHFR gene and by Baserga and Benz [1992] on the β-globin gene, has established that mutations giving rise to transcription termination codons destabilize the cognate mRNA. Maquat and her colleagues have conducted an extensive series of studies of the mechanism by which nonsense codons, which must be in an exon in frame with the translation initiation codon, can mediate a reduction in the stability of the triosephosphate isomerase transcript in the nucleus [Cheng et al., 1994]. Although details remain obscure, it appears that the chain-terminating codon interacts, via one or more proteins, with a nearby intronic sequence within the context of the normal chain terminating codon, to mediate a reduction in the abundance of the nucleus-associated cognate mRNA. The fact that suppressor tRNAs can restore nuclear mRNA levels to normal implies that transport of the transcript out of the nucleus is coupled to ongoing translation in the cytoplasm. Consequences of this process can include an enrichment for alternatively spliced transcripts that do not possess the nonsense codon (exon skipping). As noted by Connor et al. [1994] in their study on the linkage between RNA processing and RNA translatability in the mouse immunoglobulin μ heavy chain, one way to reconcile the various published reports is to invoke a nuclear scanning process that recognizes inappropriate translation termination codons and initiates degradation of the transcript.

Regulation of the stability of the MRP/PLF transcript in the MEFs, and other reported examples of unstable nuclear transcripts, does not appear to depend upon the presence of a termination codon. For example, in TE-85 osteosarcoma cells transfected to produce Ha-ras under the control of the metallothionein promoter, nuclear levels of processed fibronectin mRNA were reduced by a post-transcriptional, post-splicing mechanism when expression of the Ha-ras gene was induced [Chandler et al., 1994]. The transcript of the *Drosophila* Hsr-omega-n gene is exclusively nuclear and undergoes nuclear turnover; the levels of Hsr-omega-n RNA are modulated by a variety of factors, including heat shock, that affect both transcription and turnover [Hogan et al., 1994]. Actinomycin D, but not inhibitors of protein synthesis, blocked this degradation, suggesting the possibility that ongoing transcription, or the presence of a short-lived RNA, was involved. A comparable observation has been made for the RIIβ mRNA in rat sertoli cells [Knutsen et al., 1992]. Kiledjian and Kadesch [1991] studied liver/bone/kidney alkaline phosphatase gene expression in osteoblast-like (Saos-2) and non-osteoblast (HepG2) cells and concluded that in the HepG2 hepatoblastoma cells, which express 1,000-fold less of the mRNA than the Saos-2 cells, the nascent transcript is specifically destabilized.

CONCLUDING REMARKS

As an explanation for our results, and perhaps some others in the literature, we suggest that export of the mRNA in question from the nucleus is aborted and as a consequence the mRNA is consigned to a default pathway that results in its degradation. A corollary of this hypothesis is that transcripts made from DNA transfected into the cells by calcium phosphate are not subject to the same translocational mechanism and export controls as the endogenous transcripts. A precedent for such a model comes from a report that transcripts of adenovirus late genes are dependent on the E1B 55-kDa protein for effective transport through nuclear compartments to

the cytoplasm [Leppard and Shenk, 1989]. Also, the HIV-1 trans-activator Rev product appears to facilitate the translocation of intron-containing HIV-1 RNAs to the cytoplasm by inhibiting their degradation in the nucleus [Malim and Cullen, 1993]. Perhaps an analogous protein, required for export of the MRP/PLF transcript, is not expressed in MEFs but is expressed in the immortal 3T3 cells.

It remains unclear whether the change that occurs in the MEFs that generates the immortal 3T3 cells is genetic or epigenetic [Denhardt et al., 1991; Rittling and Denhardt, 1992]. Does it involve a change (mutation) in DNA sequence, or a change in an homeostatic regulatory system? Whatever it is, it seems to give rise with some consistency to cell lines with comparable properties when passaged under similar conditions. Since altered expression of a number of genes may accompany expression of the immortal phenotype, one or more changes in a basic regulatory mechanism connected with nuclear structure such as we describe here seems likely.

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