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The mitogen-regulated protein/proliferin transcript is degraded in primary mouse embryo fibroblast but not 3T3 nuclei: Altered RNA processing correlates with immortalization

(post-transcriptional control/RNA degradation/reverse transcriptase-PCR quantification)

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ABSTRACT **An understanding of what changes occur in the control of gene expression when mammalian cells "spontaneously" immortalize is important to our knowledge of how cancer develops. We describe here an alteration in regulation that occurs when primary mouse embryo fibroblasts (MEFs) are immortalized according to a 3T3 regimen. Mitogen-regulated protein/proliferin mRNA is undetectable in northern blots of RNA from (mortal) MEFs, whereas it is readily detected in immortal 3T3 cell lines derived from the MEFs. Incompletely processed nuclear transcripts of the mitogen-regulated protein/proliferin gene can be detected in MEF RNA preparations by northern blotting and reverse transcriptase polymerase chain reaction analyses, although at roughly half the abundance observed in 3T3 cells. We hypothesize that some attribute of the primary unprocessed transcript determines its assignment to this unique degradative pathway. These results reveal that during passage of MEFs according to a 3T3 regimen the ability of the primary cells to suppress the expression of certain genes by degrading the nuclear transcript is lost concomitantly with immortalization.**

Mouse embryo fibroblasts (MEFs) cultured in medium containing serum pass through a crisis period and give rise to immortal cell lines whose properties reflect the specific regimen used to pass the cells (1, 2). This spontaneous immortalization of primary cultures of rodent cells, which occurs infrequently in human or avian cells, is accompanied by reproducible changes in gene expression (3). We are interested in discovering the underlying basis for these changes in gene expression in order to understand what distinguishes mortal from immortal cells.

Mitogen-regulated protein [MRP; also called proliferin (PLF)] is a secreted, glycosylated, 34-kDa mouse protein of unknown function but is a member of the prolactin/growth hormone family. MRP was described in 1980 (4) as a protein whose synthesis by 3T3 cells was enhanced by serum, epidermal growth factor, and fibroblast growth factor. PLF was described in 1984 (5) as a prolactin-related protein whose mRNA was induced by serum in quiescent 3T3 cells. Since these two names refer to the same protein (6, 7), we will use the designation MRP/PLF. In the mouse MRP/PLF is produced only by the trophoblastic giant cells of the placenta during midgestation (8), and it is recognized by the cation-independent mannose 6-phosphate receptor [when the protein bears mannose 6-phosphate (9)] and by a second receptor found on cells in the placenta, uterus, and mammary gland of pregnant mice (J. T. Nelson and M. Nilsen-Hamilton, personal communication). The only known function of MRP/PLF (specifically MRP/PLF1 and MRP/PLF2) is the inhibition of myogenic differentiation of C3H/10T½ cells by a mechanism requiring inactivation of a CArG-binding factor necessary for transcription driven by myogenic-specific promoters (11, 12). Three of the estimated five or six copies of the MRP/PLF gene in the mouse genome have been partially characterized. The research reported here was performed with MRP/PLF3 (13, 14); however, as there is no evidence suggesting a significant difference between the members of this closely related family of genes, we refer throughout this work simply to MRP/PLF.

Although the MRP/PLF genes are transcribed in proliferating MEF cells as judged from nuclear run-on studies, cytoplasmic mRNA cannot be detected, whereas it is abundant in proliferating 3T3 cells derived from the MEFs (15). Efforts to identify an alteration in the genomic MRP/PLF DNA, such as amplification, altered distribution of DNase-hypersensitive sites, or changes in the methylation of CG dinucleotides, were unsuccessful (13). The implication of these studies, that in primary MEFs expression of MRP/PLF is extinguished at the post-transcriptional level, gave rise to this study in which we demonstrate that the reason for the lack of MRP/PLF expression is that in the MEFs the MRP/PLF transcript is rapidly and specifically destroyed.

MATERIALS AND METHODS

Cell Culture. Primary MEFs were prepared from 14- to 16-day CD-1 mouse embryos. The embryos were minced, trypsinized, and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum in humidified 5% CO_2. Cells were passed every 3–4 days at 1.7×10^4 cells per cm^2. Swiss 3T3 cells were obtained from the American Type Culture Collection or were prepared from MEFs by sequential passage as above until the 3T3 cell line was established (3). In the experiments described here, cells were refed with fresh medium containing 10% calf serum 12–18 hr before harvest, when they were 70–90% confluent.

Plasmid Constructs. The pMRPLuc construct was made using the pXP1 plasmid (16), which contains the *Photinus pyralis* luciferase gene (ATCC no. 37576). The MRP/PLF3 promoter [nt −1101 to +64 (14)] was cloned into the *Sma* I site of pXP1. The plasmid pCMVMRP was made by cloning most of the MRP/PLF3 cDNA plus 3′ flanking DNA that included the poly(A) site downstream of the cytomegalovirus (CMV) promoter in pCMVCAT (17) from which the simian virus 40 and chloramphenicol acetyltransferase (CAT) sequences had been removed. pCMVvimentin was constructed similarly, starting from the human vimentin cDNA (18).

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Abbreviations: CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; MEF, mouse embryo fibroblast; MRP, mitogen-regulated protein; PLF, proliferin; RT-PCR, reverse transcriptase polymerase chain reaction.

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DNA Transfection and RNA Analysis. 3T3 and primary MEF cells were plated at 8.9×10^3 cells per cm^2 and transfected 1 day later by calcium phosphate coprecipitation 3 hr after refeeding (19). To each 60-mm dish was added 5 μg of pMRPLUC, 5 μg of a β-galactosidase reporter plasmid driven by the *opn* promoter [−740 to +79 (20)], and 15 μg of pGEM plasmid DNA. After 16–18 hr the 3T3 and MEF cells were shocked with glycerol for 1–1.5 min and about 20 sec, respectively, and then incubated in fresh medium. Cells were lysed 48 hr later and assayed for luciferase using a Promega procedure and for β-galactosidase (21). RNA was prepared from cells or nuclei with acidic guanidinium thiocyanate/phenol/chloroform (22) or the TRI reagent (Molecular Research Center, Cincinnati). For northern blot analysis, 15 μg of RNA was loaded per lane of an HCHO/agarose gel, and after electrophoresis the RNA was transferred onto a GeneScreen*Plus* nylon membrane, which was exposed to UV radiation and then hybridized to 5×10^6 cpm of the different probes in 50% formamide.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). RT reactions were carried out using MMLV-RT and random hexamer primers. The RNA used for cDNA synthesis was pretreated with RNase-free DNase to eliminate genomic DNA contamination; the efficiency of cDNA synthesis ranged around 40–50%. RT reactions were carried out at 37°C for 60 min, after which the RT was denatured. PCR primers were chosen using the PCRPRIM program (23) and used at a concentration of 20 μM with deoxynucleoside triphosphates at 200 μM each; 5 μCi (1 Ci = 37 GBq) of [α-^{32}P]dCTP was included in the reaction, and the $MgCl_2$ concentration was 1.5 mM in a final volume of 100 μl. Conditions of PCR amplification with the *Taq* polymerase were as follows: for the actin control and the 5′ end of the MRP/PLF gene, 94°C for 30 sec, 61°C for 1 min, and 72°C for 1 min; for the 3′ end of the MRP/PLF gene and the mRNA, 94°C for 30 sec, 51°C for 1 min, and 72°C for 1 min. Samples were removed every other cycle and electrophoresed on a 1% agarose/0.5% Nusieve agarose gel in Tris-acetate buffer containing ethidium bromide. The DNA bands were removed, melted in 1.5-ml Eppendorf tubes in 500 μl of distilled water, and transferred to a scintillation vial with 10 ml of Ecolite scintillation fluid, and the amount of radioactivity determined.

RNase Protection Assays. MEF and 3T3 cells were transfected with 10 μg of pCMVMRP together with 10 μg of pCMVvimentin as a control; 72 hr after transfection the cells were harvested and total RNA was purified. The *Nco* I–*Mae* I fragment of pCMVMRP containing 319 nt of CMV sequence and 383 nt of MRP sequence was cloned into pGEM3ZF+ (see Fig. 5). Transcription of this plasmid with SP6 polymerase in the presence of [α-^{32}P]UTP yielded the antisense probe. The RNase protection was performed with 5×10^5 cpm of ^{32}P-labeled probe per reaction using 10 μg of RNA at 45°C for 16 hr (22). The hybridized RNA species were incubated with 500 units of RNase T1 and 12 μg of RNase A per reaction, and the resulting products were electrophoresed.

RESULTS

The Mature MRP/PLF Transcript Is Found in 3T3 but Not MEF Cells. Fig. 1 shows a northern blot of six preparations of RNA analyzed with four different probes: the complete MRP/PLF cDNA (Fig. 1*A*), a β-actin probe (Fig. 1*B*), a portion of MRP/PLF intron 2 (Fig. 1*C*), and a portion of MRP/PLF intron 4 that also included a short segment of exon 4 (Fig. 1*D*). To distinguish nuclear and cytoplasmic localizations, cells were gently lysed and nuclei were separated from the cytoplasm; unfortunately, during the lysis procedure necessary to maintain intact nuclei there was some unavoidable RNA breakdown and continued processing. The pair of lanes on the right in each panel shows total cellular RNA isolated by standard procedures that minimized RNA breakdown. Mature MRP/PLF transcripts are detected in 3T3, but not MEF, nuclei and cytoplasm. In the nucleus of 3T3 cells, and to a lesser extent in MEF cells, the MRP/PLF cDNA probe also revealed transcripts larger than the mRNA. Probes for the two introns confirmed the existence of higher molecular weight transcripts of the MRP/PLF gene in the MEFs, but at a lower abundance than observed in 3T3 cells.

Analysis of MRP/PLF Promoter Activity in MEF and 3T3 Cells. Two types of experiments were performed to ascertain the activity of the MRP/PLF promoter. Nuclear run-on experiments (15) indicated approximate equality of transcription of the gene in MEF and 3T3 nuclei. Additional evidence that the promoter could function in MEF cells came from transient transfection studies (Fig. 2) employing a luciferase reporter gene whose transcription in pMRPLuc was driven by a sequence that included 1.1 kb of the MRP/PLF3 promoter and 60 nt of the 5′ untranslated region (14). This plasmid carries the simian virus 40 poly(A) signal immediately upstream of the site into which the promoter segment was cloned in order to minimize transcription through this region initiated by upstream promoters (16). pMRPLuc was

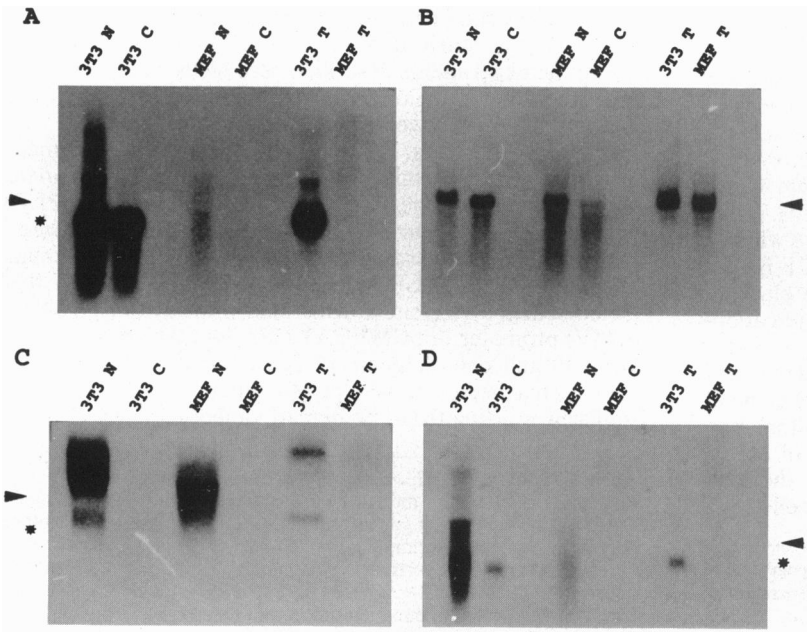

FIG. 1. Northern blot analysis of the abundance of MRP/PLF transcripts in 3T3 and MEF cells. (*A*) Autoradiogram of a blot probed with a MRP/PLF cDNA probe. The filter was stripped by boiling the membrane in 0.1× SSC/1% SDS (1× SSC = 0.15 M NaCl/15 mM sodium citrate), and subsequently reprobed first with a β-actin probe (*B*) and then with a 600-bp *Nco* I–*Acc* I fragment composed mostly of intron 4 sequence but with a bit of the 3′ end of exon 4 also, which may account for the signal in the position of the MRP/PLF mRNA in the 3T3 lanes (*D*). The blot in *C* has the same RNA samples as in the other panels but was run using larger wells and was probed with a 1.8-kb *Bam*HI–*Pst* I fragment contained within intron 2. Both blots were stripped and reprobed for the 18S RNA, whose position is indicated by the arrowhead. N, nuclear RNA; C, cytoplasmic RNA; T, total RNA. An asterisk indicates the position of MRP/PLF mRNA.

transfected into MEF and 3T3 cells by calcium phosphate coprecipitation. To normalize for transfection efficiencies, a plasmid with a β-galactosidase reporter gene transcribed from the osteopontin promoter (20) was used. Whereas the amount of luciferase activity generated by the promoterless "control" pXP1 was not significantly different from the background levels seen in mock-infected cells, the MRP/PLF promoter-driven construct exhibited considerable luciferase activity, ranging from 4-fold to 9-fold above background in the MEF and 3T3 cells, respectively. This result suggests, but does not prove, that the MEFs have the capacity to transcribe at least one of the endogenous MRP/PLF genes but are unable to convert the primary transcript into a stable mRNA. Our working hypothesis is that some as-yet-unidentified mechanism targets the MRP/PLF transcript in the nucleus of the MEF cell for rapid degradation.

PCR Analysis of MRP/PLF Transcripts in MEF and 3T3 Cells. Because the low abundance of the MRP/PLF transcript in the MEFs made quantitation by northern blotting or RNase protection assays difficult (refs. 13 and 14; Fig. 1), we turned to the more sensitive RT-PCR method of analysis. Pairs of primers were synthesized that bracketed exon–intron boundaries near the 5′ and 3′ ends of the gene as diagramed in Fig. 3*A*. When used to amplify sequences from genomic MEF and 3T3 DNA, these primers generated molecules of the expected size in each case (data not shown), confirming their functionality and the absence of major structural changes in the MRP/PLF genes in these two cell types. The lanes in Fig. 3*B* show typical products generated from two RNA preparations using the various pairs of primers. Comparison of the fluorescent band intensities in lanes 3 and 7 (MEF RNA) with lanes 1 and 5 (3T3 RNA), respectively, suggests that there are comparable amounts of unprocessed transcript in MEF and 3T3 cells. When primers E1 and E5 were used with cDNA made from 1 μg of total nuclear RNA, processed MRP message could be reproducibly detected only in 3T3 cells (lane 9). The absence of product in the reactions lacking RT (even-numbered lanes) documents that cellular DNA is not the template. The amplified products shown in lanes 13 and 15, which were generated with primers to the β-actin coding sequence, demonstrate that this gene was equally represented in the MEF and 3T3 RNA preparations.

To further characterize the MRP/PLF transcript in the MEF nucleus, we undertook to estimate the relative amounts of different segments of the transcript in the nuclei of these cells in comparison to 3T3 cells (24). Portions were removed from the PCR after every other cycle (usually between cycles 20 and 40), and the amount of radioactivity incorporated into the specific DNA fragment, purified from a gel, was determined. During the amplification process there exists a window in which the incorporation of radioactivity (from [α-^{32}P]dCTP) into product can be seen to be truly exponential. After n cycles of amplification, the number of mol of DNA per μl of reaction, (N_n), can be calculated by N_n = [cpm/μl]/[y(cpm/mol)], where cpm/μl is the radioactivity in the gel-purified DNA fragment, cpm/mol is the specific activity of the [α-^{32}P]dCTP precursor, and y is the number of cytosines in the product. The linear portion of the semilogarithmic plot of N_n vs. cycle extrapolated to the y axis yields N_o, the mol of cDNA per μl present at the start of the reaction according to $\log N_n = n\log(\text{eff}) + \log N_o$, where eff is the efficiency of the amplification, ideally 2 (24). Fig. 4 shows typical plots obtained with the three different primer sets described in the legend to Fig. 3. These experiments generated the data in Table 1, establishing that mature mRNA was

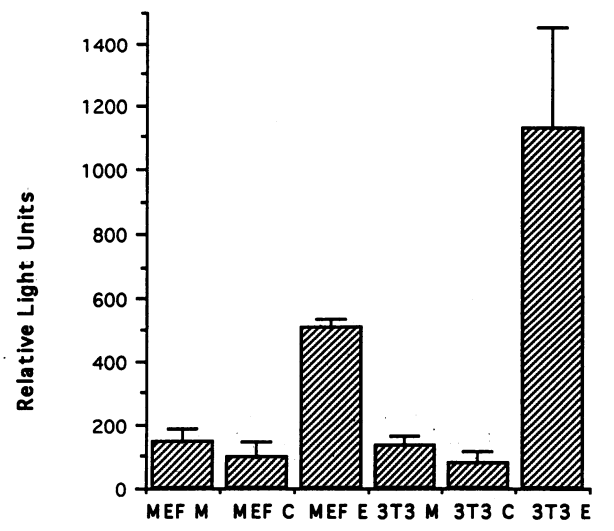

FIG. 2. Analysis of the MRP/PLF3 promoter in MEF and 3T3 cells. A MRP-luciferase construct was made by cloning 1.1 kb of the MRP/PLF3 promoter plus 60 nt of the 5′ untranslated sequence into the *Sma* I site of pXP1 after blunting the *Eco*RI and *Pst* I ends (see figure 1 of ref. 14). Shown are the results of an assay for luciferase after a transient transfection of 3T3 and MEF cells. M, mock transfected; C, transfected with parent plasmid pXP1; E, transfected with pMRPLuc. Ten microliters of the lysate was used to determine luciferase activity, which was normalized against β-galactosidase activity generated by a cotransfected osteopontin-β-galactosidase construct. The results shown are from three independent assays and are expressed as mean ± SE.

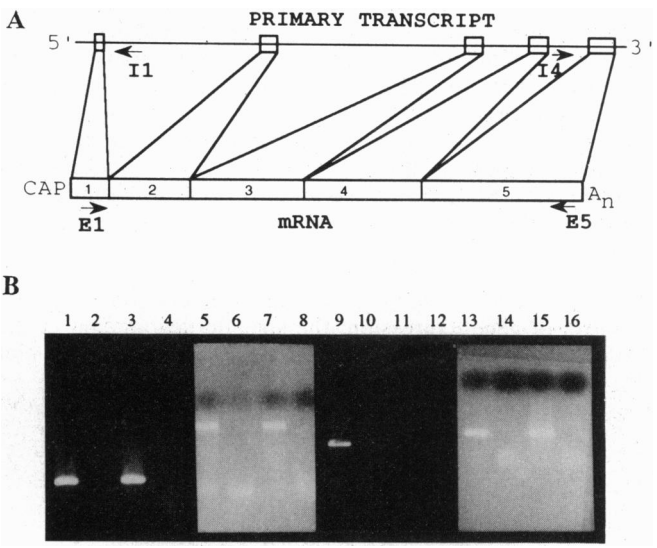

FIG. 3. Analysis of transcript levels in MEF and 3T3 cells by RT-PCR. (*A*) The primary transcript of the MRP/PLF gene and its 5-exon–4-intron organization is illustrated above a representation of the processed MRP/PLF mRNA. E1, I1, I4, and E5 represent PCR primers whose sequences are as follows: E1, 5′-CCCTTCTTCGATTCAACCATG-3′; I1, 5′-AGACACTGCTGCATACTCTAGG-3′; I4, 5′-ACAACAAACCCATCTCAGG-3′; E5, 5′-CATGTAACACTTCAGGACG-3′. PCR primers for the mouse β-actin gene were as follows: 5′ β-actin, 5′-GCCAGGTCATCACTATTGG-3′; 3′ β-actin, 5′-AGTAACAGTCCGCCTAGAAGC-3′. (*B*) Agarose gel electrophoresis of the RT-PCR reaction carried out with nuclear RNA. Each RT reaction was divided into five equal portions, four of which were used for quantification of the 5′, 3′, mRNA, and β-actin sequences. Odd-numbered lanes are RT-PCR reactions with RT; even-numbered lanes are controls in which RT was omitted. Reactions in lanes 1, 2, 5, 6, 9, 10, 13, and 14 were performed with 3T3 nuclear RNA; those in lanes 3, 4, 7, 8, 11, 12, 15, and 16 were performed with MEF nuclear RNA. Lanes 1–4, 30 cycles using primers E1 and I1, which target the 5′ end of the primary transcript (size of the product, 300 bp); lanes 5–8, 35 cycles using primers I4 and E5, which target the 3′ end of the primary transcript (size of the product, 1000 bp); lanes 9–12, 30 cycles using primers E1 and E5, which target the processed transcript (size of the product, 680 bp); lanes 13–16, 25 cycles using the 5′ and 3′ actin primers (size of the product, ≈400 bp).

essentially absent in MEF cells and that the abundance of the primary transcript in these cells was about half that in 3T3 cells.

Accumulation of MRP/PLF mRNA Transcribed from a cDNA Recombinant Vector. By transfecting a MRP/PLF cDNA expression plasmid into MEF and 3T3 cells, we have found that the MRP/PLF message accumulates to comparable levels in both. Expression of the MRP/PLF mRNA was engineered by placing the MRP/PLF cDNA plus 3′ flanking sequence [including the poly(A) signal] under the control of the CMV promoter. This plasmid, shown in Fig. 5*A*, was transfected into MEF and 3T3 cells, and the abundance of MRP/PLF mRNA was assayed by RNase protection. As a control for transfection efficiency, an analogous construct encoding the stable vimentin mRNA was cotransfected, and the levels of the vimentin message were assayed simultaneously. The results of an RNase protection assay are shown in Fig. 5*B*. As diagramed in Fig. 5*C*, the endogenous MRP/PLF message protects a 383-nt fragment, whereas RNA transcribed from the transfected MRP/PLF construct protects a fragment of 479 nt that includes the MRP/PLF sequence plus some 5′ untranslated sequences in the CMV element. The CMV-vimentin transcript protects only the CMV 5′ untranslated sequence; the multiple species around 96 nt reflect the use of several start sites by the CMV promoter.

In this experiment endogenous MRP/PLF mRNA (at 383 nt) was again evident in 3T3 cells but not in MEFs. When the amount of the CMV-generated MRP/PLF transcript (at 479 nt) was normalized by densitometry for transfection efficiency by comparison to the CMV-vimentin transcript, the calculated levels of MRP/PLF transcript in the 3T3 and MEF cells were 100 and 123 arbitrary units, respectively. Clearly, there is not a large difference in the levels of this transcript that accumulate in the two cell types. We conclude that MEFs are able to transcribe and accumulate the intronless MRP/PLF mRNA to the same extent as 3T3 cells.

DISCUSSION

Our experiments highlight a poorly understood means of control of gene expression: the specific degradation of the MRP/PLF transcript in the MEF nucleus. Northern blot analyses with cDNA and intron probes (Fig. 1) and a study of the MRP/PLF promoter (Fig. 2) indicated that the gene could be transcribed in the MEF cells; however, mature mRNA was not detectable. The RT-PCR analysis (Figs. 3 and 4) confirmed the presence of unprocessed MRP/PLF transcripts in MEFs. The apparent excess of 5′ over 3′ ends (Table 1) could indicate a true excess or could be an artifact caused by the smaller size of the PCR product representing the 5′ end. A MRP/PLF mRNA transcribed from a cDNA "minigene" under the control of the CMV promoter that contained all but 60 nt of the 5′ end was stable in MEFs. The remaining 60 nt from the 5′ end of the mRNA were present in the promoter construct shown to be functional in MEFs

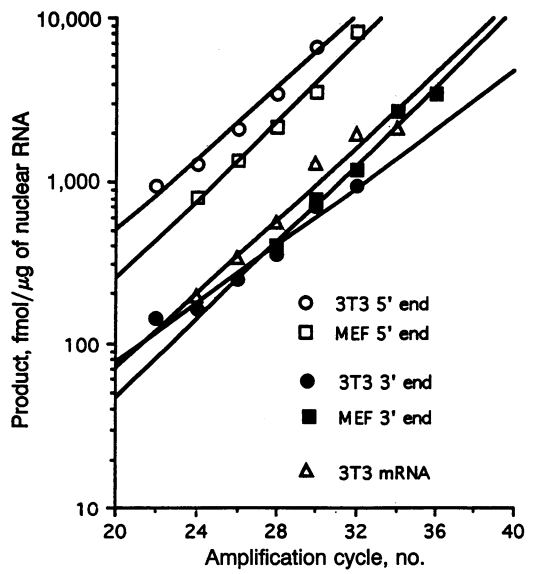

FIG. 4. Accumulation of the PCR product during the exponential phase of the amplification reaction. The amount of the PCR product (determined from the incorporated radioactivity) is plotted against the cycle number during the exponential phase of the reaction. At earlier cycles no radioactivity is detectable, and at later cycles the reaction begins to plateau. Straight lines were fitted to the points and extrapolated to determine the *y* axis intercept at cycle zero.

Table 1. Abundance of MRP/PLF transcripts in the nucleus

Cells	5′ end	3′ end	mRNA
3T3	46 ± 25	13.6 ± 4.4	2.3 ± 0.5
MEF	25 ± 6.3	5.2 ± 1.4	—

Data are expressed as fmol/μg of nuclear RNA (mean ± SE). They are the results of three independent experiments with different preparations of nuclear RNA using the appropriate pair of primers for each portion of the primary transcript or the mRNA (Fig. 3*A*). Given 5 pg of RNA in the nucleus, 1 fmol/μg would correspond to about 3000 molecules per nucleus. Processed mRNA could not be reliably detected in the MEFs. Attempts to assign a lower limit of detectability using a model system were frustrated by a lack of reproducibility when the number of amplification cycles approached 40. The presence of a few molecules of MRP/PLF mRNA in the MEFs could result from the inability of the cell to absolutely block the generation of mature message or the presence of a few cells in the population that had acquired the ability to process the transcript (25).

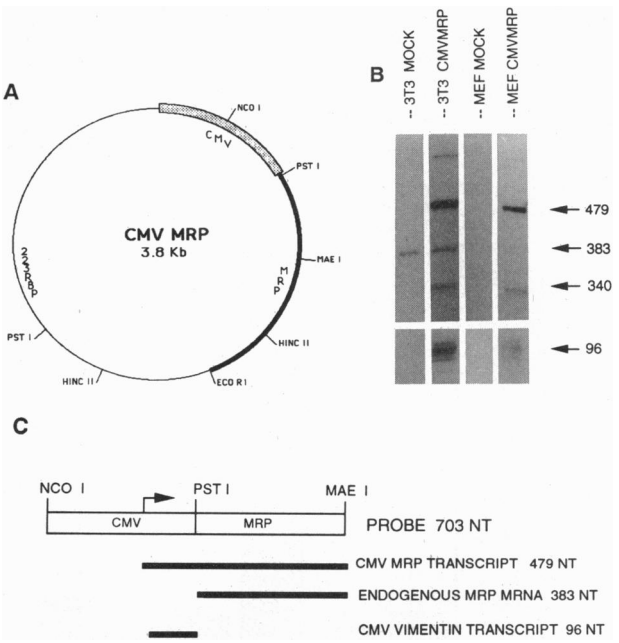

FIG. 5. RNase protection analysis of RNA transcribed from an expression vector containing the MRP/PLF3 cDNA. The complete cDNA sequence is present except for 60 nt at the 5′ end of the untranslated region. (*A*) Map of the pCMVMRP plasmid. (*B*) Autoradiogram of an acrylamide gel analysis of the protected fragments of RNA from cells transfected with pCMVMRP plus pCMVvimentin. The positions of the bands corresponding to the transcript from the transfected MRP/PLF construct (479 nt) and the endogenous MRP/PLF RNA (383 nt) are indicated. The 340-nt species is of unknown origin, whereas those around 96 nt result from protection of the CMV portion of the probe by RNA generated from the pCMVvimentin included as an internal control. (The central portion of the gel contained no bands and is omitted to save space.) (*C*) Cartoon showing the predicted sizes of the protected fragments.

(Fig. 2). We conclude that the mature mRNA is unlikely to contain a sequence element that by itself destabilizes it in MEF cells. It may be that the primary MRP/PLF transcript cannot be correctly processed in the MEFs and is therefore consigned to a default pathway that results in the elimination of such transcripts. Alternatively, an unrecognized feature of the transcript may sensitize it to nuclease attack in the MEFs but not in 3T3 cells.

It has long been known that much of the newly synthesized RNA in most eukaryotic cells is destined to be destroyed in the nucleus, and there have been a few reports of specific genes that might be regulated at this level. Excised introns are rapidly catabolized, and the importance of intron excision for the proper processing and transport of certain transcripts has been demonstrated, for example, for simian virus 40 late transcripts, where the absence of introns somehow allows degradation of the transcript in the nucleus (26). Resting (G_0) peripheral T cells, in contrast to mitogen-activated cells, possess very low levels of eukaryotic initiation factor 2α mRNA because the primary transcript is rapidly degraded (27). Androgens appear to regulate expression of some target genes in seminal vesicles by enhancing the stability of the primary transcript in the nucleus (28). Interleukin 4 blocks interleukin 1α induction of granulocyte/macrophage colony-stimulating factor in B lymphocytes by destabilizing the nuclear transcript (29). Human peripheral blood lymphocytes possess high levels of calcyclin precursor transcripts, but no processed mRNA; mature mRNA is present, however, in leukemic blast cells (30). The proliferating cell nuclear antigen gene is transcribed in senescent human diploid fibroblasts, but mature mRNA does not accumulate as it does in young, proliferating cells (31).

Our work raises two issues concerning the process of immortalization. One is the relevance of MRP/PLF expression *per se* and the other is the more general question of the importance of alleviating this mechanism of suppression of gene expression (i.e., degradation of specific transcripts in the nucleus). With regard to the first point, some immortal mouse lines (e.g., JB6, 3T12, B16F10) do not produce MRP/PLF, indicating that it is not a necessary correlate of immortalization; furthermore, MRP/PLF synthesis has been detected in senescent, mortal cells in culture (25). However, we cannot exclude that MRP/PLF affects gene expression uniquely in the 3T3 cells in an autocrine manner to confer immortality. A definitive conclusion regarding its role in the immortalization of MEFs in serum-containing medium by a 3T3 regimen will come only from experiments in which MRP/PLF expression is specifically suppressed.

With regard to immortalization, the mechanism(s) by which mouse cells *spontaneously* escape senescence remains elusive. Genetic (mutational changes in DNA sequence) and epigenetic (changes in gene expression resulting from non-mutational events) causes have been championed (see refs. 32 and 33 for recent discussions of this issue). We think it is unlikely that MRP/PLF is the only gene whose expression is regulated by degradation of nuclear transcripts, and, if so, then one or more of these yet-to-be-identified genes whose expression is controlled in this way may well be important to the immortalization process, at least in this paradigm. Like cell transformation, immortalization of different cell types appears to follow different pathways. It will be interesting to discover how many of these pathways include the regulatory change we have identified here.

The general importance of this mechanism of regulation of gene expression—targeted elimination of specific transcripts in the nucleus—and how it is controlled remain to be determined. It is important to find out the extent to which this mechanism of suppression of gene expression is used to abrogate expression of genes that are normally expressed only in a very limited subset of tissues. MRP/PLF is ordinarily expressed only during early embryonic development in the giant trophoblast cells. Is suppression of expression in adult tissues dependent upon degradation of the primary transcript in the nucleus? Is expression in the trophoblast achieved in the same way that it is in 3T3 cells? The work of Parfett (10), which shows a close correlation between the ability of various nonmutagenic tumor promoters to induce morphological transformation in C3H/10T½ cells and to induce MRP/PLF expression, may be evidence for an "epigenetic" control over MRP/PLF expression. Further study of this process of regulating gene expression independently of transcription is clearly warranted.

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