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Abstract
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Characterization of a cDNA Clone Encoding Murine Mitogen-Regulated Protein: Regulation of mRNA Levels in Mortal and Immortal Cell Lines

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Mitogen-regulated protein (MRP) is secreted by certain immortal murine cell lines (Swiss 3T3, BNL) stimulated with serum or particular growth factors. We have identified a cDNA clone that encodes part of the protein and have confirmed that MRP is closely related to, if not identical to, the prolactin-related protein designated proliferin. MRP is not produced by primary mouse embryo fibroblasts to nearly the same extent as it is produced by many immortal or transformed lines. Control of expression of this protein by growth factors is achieved both by regulating the extent of transcription and by regulating the processing of the protein.

Mitogen-regulated protein (MRP) was described by Nilsen-Hamilton et al. (12) as a [^{35}S]methionine-labeled family of glycoproteins (M_r, 30,000 to 38,000) released by Swiss mouse 3T3 cells some 18 to 28 h after stimulation with growth factors (serum, fibroblast growth factor, epidermal growth factor); platelet-derived growth factor and the tumor promoter 12-tetra-decanoylphorbol-13-acetate were shown subsequently to be effective at stimulating release also. Evidence for glycosylation includes the facts that treatment with endoglycosidase H reduces the apparent molecular weight substantially and that the protein synthesized in the presence of tunicamycin has a lower M_r of about 22,000. An antiserum raised against purified MRP has been used to identify the protein unambiguously.

Not only mitogens, but also substances that increase intralysosomal pH (NH_4Cl, chloroquine, and the ionophores monensin and nigericin) enhance the production of MRP (14). This suggests that MRP levels are, in part, regulated at the level of protein degradation, since these latter substances inhibit degradation of lysosomal proteins. In agreement with this is the observation that the ionophore-stimulated release of MRP is not blocked by actinomycin D, in contrast to growth factor-induced release (M. Nilsen-Hamilton and R. T. Hamilton, unpublished data). Proteins known to be secreted by fibroblasts include plasminogen activator, collagenase, fibronectin, procollagen, and major excreted protein (13). Proteins for which there is evidence (in other cell types) that the secreted quantities are regulated by intracellular degradation include prolactin, parathormone, and procollagen (2).

We have used an antiserum raised against MRP (R. T. Hamilton and M. Nilsen-Hamilton unpublished data) to screen a λ*gt*11 expression library (18) for clones able to code for one or more epitopes found on MRP. Mouse BNL cells (15) were used as the source of poly(A) mRNA for cloning, since among the cell lines tested it produced the most MRP. To provide initial enrichment for poly(A) mRNA molecules coding for MRP, the cytoplasmic poly(A) mRNA extracted from a growing population of mouse BNL cells was fractionated by velocity sedimentation in a sucrose gradient containing formamide. We recovered the RNA in separate pools and examined it by in vitro translation and immunoprecipitation to identify the fraction most enriched for MRP mRNA. The cDNA synthesized from that fraction by the method of Land et al. (6) was used to construct a λ*gt*10 library (λ*gt*10 provided by T. Huynh and R. Davis, Stanford University). The inserts were subsequently subcloned into λ*gt*11. Approximately 5×10^4 recombinant λ*gt*11 plaques were screened with anti-MRP antibody, and positive plaques were identified at a frequency of one in 5,000. Three positive plaques were chosen for DNA preparation and *Eco*RI restriction; each of these presumptive λ*gt*11 MRP clones contained an insert of approximately 230 base pairs.

In vitro translation of hybrid-selected mRNA was used to verify the identity of two putative MRP clones and to discriminate against the possibility that the cDNA clones recovered by the immunoscreening procedure coded for an unrelated, but cross-reacting, peptide. Preimmune serum did not produce a positive reaction. Hybrid-selected (11), complementary mRNA was translated in a reticulocyte lysate; the resulting [^{35}S]methionine-labeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5). A single major translation product was selectively enriched after hybridization of mRNA to the two MRP cDNA clones immobilized on aminophenylthioether paper, and immunoprecipitation of these translation products with anti-MRP confirmed that the major translation product (M_r, 26,000) was recognized by the antiserum (Fig. 1).

The nucleotide sequence obtained for one MRP cDNA clone contained a single open reading frame spanning the entire 226-base-pair insert. Comparison of this sequence with that determined for the protein-coding region of proliferin, a serum-induced mRNA of BALB/c 3T3 cells (8, 9), revealed an exact match with nucleotide residues 379 to 604 of the corresponding cDNA clone PLF-1. The region of

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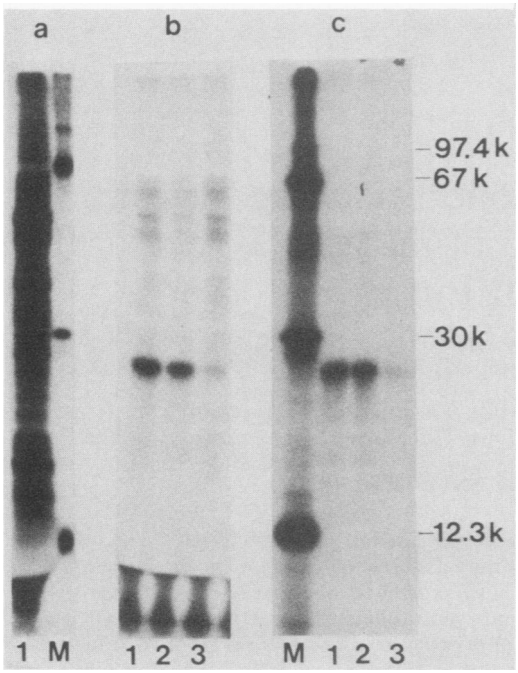

FIG. 1. Hybrid selection and in vitro translation of MRP mRNA. Shown is a fluorograph of a sodium dodecyl sulfate-polyacrylamide gel of the [^{35}S]methionine-labeled proteins produced by translation of BNL mRNA in a reticulocyte lysate. (a) Lanes: 1, total BNL mRNA; M, molecular weight markers. (b) Hybrid selection of MRP mRNA with MRP cDNA clones. Two separate MRP cDNA clones that had been identified in the antibody screening step were subcloned into M13mp8. Viral replicative form DNA was isolated, denatured, and fixed to aminophenylthioether paper (11). Lanes: 1 and 2, MRP cDNA clones; 3, M13mp8 replicative form DNA. (c) Immunoprecipitation (16) of products translated from the hybrid-selected mRNA shown in b.

overlap represents an area toward the carboxyl terminus of the peptide. This identity confirms the conclusion reached earlier on the basis of a positive antibody reaction with anti-MRP and proliferin protein generated from a eucaryotic expression vector containing the cloned proliferin cDNA (M. Nilsen-Hamilton, R. T. Hamilton, and S. Lee, manuscript in preparation).

Previous observations on the amounts of secreted MRP have revealed that Swiss 3T3 (17) and BALB/c 3T3 (1) cell lines respond differently to the addition of growth factors to the conditioned medium of growing cells. In contrast to BALB/c 3T3 and SV40-transformed Swiss 3T3 cells, addition of fibroblast growth factor to Swiss 3T3 cells stimulated them to synthesize and secrete MRP (14). To assess relationships between cytoplasmic MRP mRNA content and the ability or inability to secrete MRP, we compared several permanent mouse cell lines by Northern gel analysis, as described elsewhere (4; D. R. Edwards, C. L. J. Parfett, and D. T. Denhardt, Mol. Cell. Biol., in press), by using the 226-base-pair cDNA clone as a probe. Poly(A) mRNA from cultures growing in medium containing 10% fetal bovine serum (FBS) gave strong autoradiographic signals (Fig. 2a). The two Swiss 3T3 lines (ATCC CCL92 and CLL173) contained the greatest relative levels of MRP mRNA, whereas the two BALB/c 3T3 lines (ATCC CCL163 and CCL163.1) and the BNL line contained lesser amounts. The JB6 epidermal line (3) had no detectable MRP mRNA (data not shown). In a second experiment, in which we prepared mRNA from growing BNL, Swiss 3T3, and BALB/c 3T3 cells 20 h after replacing the culture medium with fresh medium plus 10% FBS, the levels of MRP mRNA were nearly identical in all three cell lines, suggesting that growth conditions may affect the abundance of this transcript (Fig. 2b).

Serum induction of MRP mRNA was observed when quiescent Swiss 3T3 cells maintained in medium containing 0.5% serum were stimulated with medium containing 10% FBS. A l-kilobase poly(A) mRNA that was undetectable in quiescent cells rose to substantially higher levels by 18 h after stimulation (Fig. 3a), as has been documented for proliferin in BALB/c 3T3 cells. Some message migrating slightly faster on the Northern gels could be detected in the fractions of RNA enriched in poly(A)-deficient RNA, and this species also was increased at 12 and 18 h after stimulation (Fig. 3b). The induction of MRP mRNA after 6 h of incubation in 10% FBS could be prevented by adding either of the two transcription inhibitors actinomycin D or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, suggesting that the observed increase in cytoplasmic MRP mRNA was under transcriptional control (Fig. 3c).

To extend our observations on the regulation of MRP gene expression to normal mouse cells, we also examined the induction of MRP mRNA after serum stimulation of quiescent secondary mouse embryo fibroblast (MEF) cultures prepared from 14- to 16-day-old embryos as described previously (4). Fig. 3a also shows the hybridization signals obtained with a control cDNA clone (pLP1) corresponding to an mRNA species whose abundance was constant in both MEFs and 3T3 cells regardless of the growth state of the cells. Only trace amounts of MRP mRNA could be detected in MEFs up to 28 h after refeeding with 10% FBS-containing medium (Fig. 3a). DNA synthesis began at about 12 h after serum addition (data not shown). Other mRNA species known to be induced by serum were readily observed to increase over this period with the same RNA preparations (Edwards et al., in press). Thus, failure to detect significant enrichment of MRP mRNA in cytoplasmic poly(A) mRNA after serum stimulation does not reflect general unresponsiveness in MEFs at the level of mRNA transcription or processing. Higher concentrations (20%) of serum were also unable to induce MRP transcription (data not shown).

Our demonstration that (i) there was complete sequence identity over the region shared by the MRP and proliferin cDNA clones, (ii) both hybridized to an mRNA approximately 1 kilobase in size, and (iii) the MRP mRNA was induced in response to serum stimulation of quiescent cells in culture supports the conclusion (Nilsen-Hamilton et al., in preparation) that mitogen-regulated protein and proliferin are one and the same.

Comparison of the levels of poly(A) mRNA within a group of six permanent cell lines and a culture of normal mouse embryo cells suggested two points of regulation. Cytoplasmic accumulation of mRNA was very dependent on conditions among the immortal cells studied, indicating that transcription and processing of the message are likely points for regulatory controls. This conclusion was supported in a study of transcriptional regulation of serum-induced RNAs from Swiss 3T3 fibroblasts in which both the level of MRP mRNA within the nucleus (Edwards et al., in press) and the amount of nuclear runoff transcription increased after serum stimulation of Swiss 3T3 cells (D. R. Edwards, unpublished observation). Furthermore, the induction of MRP mRNA and cellular proliferative responses are separable, since this species was undetectable in the cytoplasm of serum-

stimulated normal mouse embryo cells under conditions in which DNA synthesis and cell division occurred; also, no MRP mRNA could be detected in JB6 cells growing in 10% FBS.

Although MRP mRNA was inducible in both Swiss 3T3 and BALB/c 3T3 mouse cells, the protein was not detected in BALB/c 3T3 cells after stimulation by calf serum or purified growth factors (12, 14), pointing to possible translational or postranslational regulatory mechanisms that operate differently in these cell lines. In this regard it has been shown that lysosomal degradation of newly synthesized MRP plays a major role in determining the quantity of MRP secreted by Swiss 3T3 cells into the culture medium (14; Nilsen-Hamilton et al., submitted for publication).

It is interesting that cell lines expressing the MRP gene(s) predominate among those so far examined (BNL; NIH 3T3, 3T6, and Ehrlich ascites cells are especially good). We have observed this mRNA in five of six independent cell lines, whereas Linzer and Nathans (10) reported proliferin mRNA in C3H 10T1/2 and Krebs ascites carcinoma cells. These observations raise the possibility that mitogen-inducible expression of MRP could serve as a molecular marker for many of the cells selected to exhibit permanent growth in vitro. Absence of detectable MRP transcripts in JB6 cells indicates that MRP is not obligatorily involved in the establishment phenomenon. Investigations of the tissue specificity of MRP (proliferin) synthesis in the mouse (7) and the factors responsible for regulating its expression in vivo may provide clues to the origin of MRP expression in cell lines.

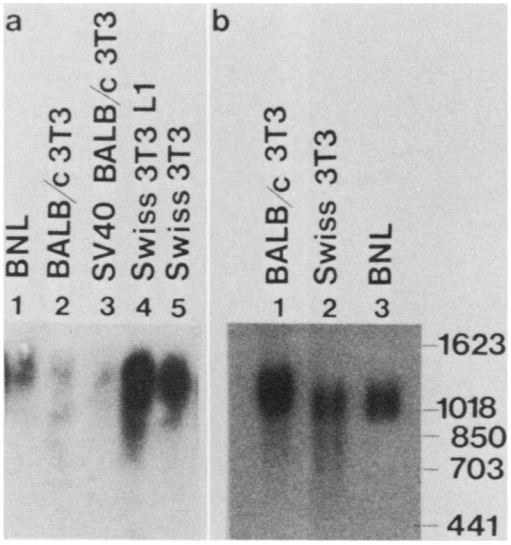

FIG. 2. MRP mRNA content of mouse cell lines. (a) Glyoxal-denatured poly(A) mRNA (4 μg in each lane) extracted from subconfluent, growing cultures of each cell line was separated by electrophoresis in 1.0% agarose and transferred to nitrocellulose. The blot was hybridized with nick-translated, denatured MRP-M13mp8 double-stranded DNA as the probe. (b) Total cytoplasmic RNA (20 μg in each lane) was isolated from subconfluent, growing cultures 20 h after a change to fresh culture medium supplemented with 10% FBS. The RNA was electrophoresed in a 1.1% agarose gel in 2.2 M formaldehyde (11) and then blotted and probed as in a. The size markers were produced by *Hae*III and *Taq*I restriction of M13mp8 and M13mp7 DNAs, respectively. Numbers on the right indicate the lengths, in nucleotides, of marker single-stranded oligodeoxynucleotides.

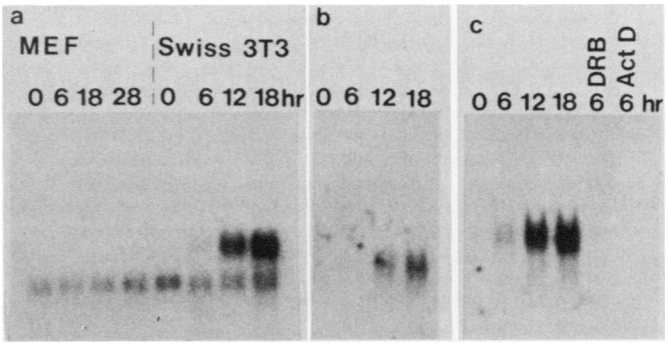

FIG. 3. Serum induction of MRP mRNA in Swiss 3T3 cells and secondary MEFs. Electrophoresis, blotting, and hybridization were the same as in Fig. 2b. (a) Poly(A) mRNA was isolated from cells at the indicated times after stimulation of quiescent cells with medium containing 10% serum. Swiss 3T3 cells and MEFs were made quiescent by growth for several days in medium containing 0.5 and 2% serum, respectively. Four micrograms of each poly(A) mRNA preparation was used per lane. The blot was also hybridized with a probe derived from a control clone (pLP1) that corresponded to an mRNA of approximately 700 nucleotides, whose abundance is invariant in cultured MEFs and 3T3 cells. (b) Swiss 3T3 poly(A)-deficient RNA (20 μg) (i.e., RNA that failed to bind to oligo(dT)-cellulose in a single binding-elution step) from cells at the same time points as in a was applied to each lane. (c) Total cytoplasmic RNA (20 μg) from Swiss 3T3 cells at various times after serum stimulation and after 6 h with (i) 10% serum plus 25 μg of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; Calbiochem-Behring, LaJolla, Calif.) per ml or (ii) 10% serum plus 2 μg of actinomycin D (Act D) per ml.

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