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EVIDENCE THAT POST-TRANSCRIPTIONAL CHANGES IN THE EXPRESSION OF MITOGEN REGULATED PROTEIN ACCOMPANY IMMORTALIZATION OF MOUSE CELLS

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Primary mouse embryo fibroblasts passed in culture go through a period of declining growth rate and then after a period of minimal proliferation begin to grow again, generating, under appropriate conditions, an immortal 3T3 line. The 3T3 cells, but not the primary mouse embryo fibroblasts, are able to synthesize and to secrete mitogen regulated protein (MRP, also known as proliferin). We report here that the level at which the change in gene expression occurs during this spontaneous immortalization process is post-transcriptional, reflecting a change in either RNA processing or transcript stability. To our knowledge, this is the first report of an alteration at the post-transcriptional level of gene expression that accompanies immortalization.

Mitogen regulated protein (MRP) comprises an immunologically related set of heterogeneously glycosylated proteins of Mr 33,000 - 37,000 that are secreted into the culture medium following mitogen stimulation of Swiss mouse 3T3 cells (1). It was independently discovered as a protein encoded in an mRNA that was inducible in quiescent BALB/c 3T3 cells by serum; this protein was called proliferin because of its sequence homology with prolactin and its perceived association with proliferating cells (2). MRP/proliferin is synthesized by established 3T3 cell lines but not by primary mouse embryo fibroblasts (3). We undertook the study reported here to determine if the acquisition of the ability to produce MRP/proliferin was regularly associated with the development of an immortal 3T3 line, and if so at what level of gene regulation

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Abbreviations: MRP, mitogen regulated protein; MEP, major excreted protein.

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this occurred. In three out of three experiments the capacity for secretion of MRP was acquired as the 3T3 line arose, and it appeared to result from a change in the processing of nuclear transcripts, or possibly the stability of the mRNA.

MATERIALS AND METHODS

Cell culture, protein labelling, immunoprecipitation. Mouse embryo fibroblast cultures were started from trypsin-treated, minced whole 14-16 day Swiss mouse embryos and grown in Dulbecco's modified MEM (4). Cells were passed every three days at 1.5×10^4 cells/cm^2, the 3T3 regimen of Todaro and Green (5). Labelling was performed with [^{35}S]methionine for two hours 18 hours after stimulation with fresh DMEM + 10% FCS as described (3); time course experiments have shown that under these conditions the amount of labeled protein in the medium is proportional to the rate of synthesis. Equal portions of the medium were first immunoprecipitated with normal rabbit serum and then with anti-MEP or anti-MRP and the immunoprecipitates collected with Protein A-Sepharose (1,6). The washed precipitates were dissolved in sample buffer and electrophoresed on 12% polyacrylamide gels in the presence of mercaptoethanol and dodecyl sulfate (7). The gels were impregnated with 2,5-diphenyloxazole, dried and fluorographed at -70°C using preflashed Kodak XAR film.

Isolation and analysis of RNA. Total cytoplasmic RNA was purified from cells at the indicated passage number (0 corresponds to what is in effect the second passage of the cells derived from the mouse embryos) and electrophoresed on a 1.1% agarose gel containing 2.2 M formaldehyde (8). The RNA was blotted onto nitrocellulose, which was then hybridized successively (with intervening stripping) with ^{32}P-labeled probes defining MEP (6), MRP (3), and 2ar (9). After annealing and washing, the blot was autoradiographed at -70°C with an intensifying screen.

Nuclear run-on analysis. Nuclei were prepared from subconfluent cells that had been stimulated 18 hours earlier with fresh medium + serum and incubated with [α^{32}P]UTP. The total nuclear RNA was extracted in guanidinium isothiocyanate with phenol at 65°C and annealed to "slot-blots" containing denatured DNA (10).

RESULTS

In the mouse the only place where MRP/proliferin has been detected is in the fetal part of midgestational placenta (11). However, in culture a variety of immortal mouse lines produce MRP. SDS-polyacrylamide gels of immunoprecipitates shown in Fig. 1a reveal that NIH 3T3 and BNL (12) cells are particularly good producers, followed by Swiss 3T6 and Ehrlich Ascites cells. Swiss and Balb/c 3T3 lines are also producers, whereas Swiss and Balb/c primary mouse embryo fibroblasts (MEFs) and their 3T12 derivatives (not shown) make no detectable MRP and have no detectable mRNA (3,13). MEP (major excreted protein) (14), which has recently been shown to be related to cysteine proteases (6),

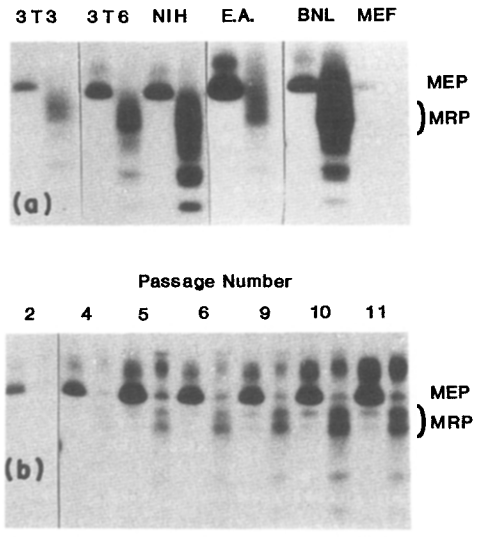


Fig. 1. SDS-PAGE analysis of MEP and MRP secreted by mouse cells in culture. Each pair of lanes represents equal portions of medium from actively proliferating cells immunoprecipitated with anti-MEP on the left and anti-MRP on the right. (See Materials and Methods for details.) MEP and MRP migrate with apparent Mrs of 39,000 and 33,000-38,000 respectively; a variable amount of a protein of around 50,000 Mr sometimes accompanies the immunoprecipitates for reasons we don't understand. (a) From right to left are media from Swiss 3T3 cells, Swiss 3T6 cells, NIH 3T3 cells, Ehrlich ascites cells, BNL cells, and primary Swiss mouse embryo fibroblasts. (b) From left to right are the media from cultured Swiss mouse embryo cells at the indicated passage level.

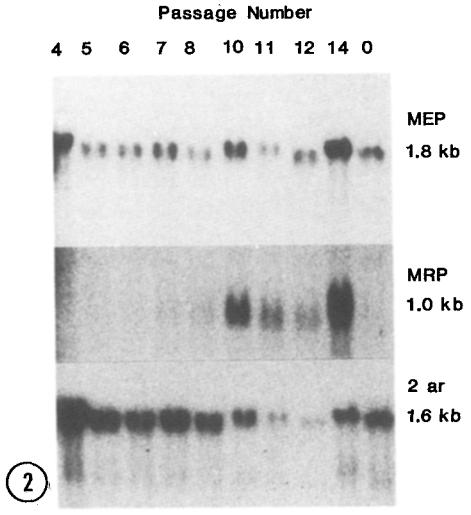
was immunoprecipitated in parallel with MRP for comparative purposes. The expression of both of these proteins is sensitive to growth conditions, and the relative amounts observed shows some variation between experiments. These results (fig. 1a) were obtained from similar numbers of cells labeled and processed in parallel to make the point that a variety of immortal mouse cell lines make MRP.

Since primary mouse embryo fibroblasts (MEFs) do not make MRP whereas certain immortal lines derived from them do, it is relevant to ask when during development of the immortal line this change occurred. Cells derived from 14-16-day old Swiss mouse embryos were passed according to the 3T3 regimen, i.e. passed every 3 days at the equivalent of $3x10^5$ cells per 5-cm plate (5). After 1-2 weeks the growth rate decreased and remained at a quite low level for several weeks before it recovered; this so-called "crisis" period usually occured around passages 4 to 8 (10-20 doublings). Quantitative analyses of this phenomenon by others have suggested that a proportion of the cells in the

starting culture develop new growth properties that enable them to survive senescence; the immortal line is not simply the result of the outgrowth of a few already immortal cells in the original population (5, 15). As the cells are passed during the crisis period the population, which remains fairly constant in size, comprises a complex, changing combination of viable and non-viable senescent cells, proliferating mortal cells and the proliferating immortal cells that eventually dominate the culture. As can be seen in fig. 1b, cells passed according to the 3T3 regimen begin to secrete MRP as they go through crisis. Cells passed according to the 3T12 regimen, during which more extensive cell contact is maintained, do not acquire the ability to make MRP (13). Other cells we have found that do not make MRP include the JB6 epidermal and B16F1 melanoma lines. When 3T3 cells are transformed with SV40 the amount of MRP produced is reduced (1).

The data presented in figure 2 monitor the appearance of MRP mRNA in the evolving 3T3 line. Early passage MEFs do not contain detectable levels of MRP mRNA in the polyA+ cytoplasmic fraction. This same blot was also probed to determine the mRNA levels for MEP and for 2ar, a TPA-inducible mRNA we are studying (9). As can be seen, these messages are present in all of the cytoplasmic polyA+ RNA preparations from cells at different passages; they vary in apparent abundance in the different preparations because their levels are very sensitive to the physiological state of the cell.

To determine whether the inability of early passage MEFs to make MRP was the result of a failure to transcribe the MRP genes we performed the nuclear run-on experiment shown in fig. 3. Nuclei were isolated from serum-stimulated MEF and 3T3 cells and incubated in vitro with [α^{32}P]UTP (10). The labeled RNA was annealed to slot-blots containing DNA (note that different amounts were loaded) complementary to various RNA species. All of the clones tested yielded similar results - that is, the genes were transcribed at comparable levels. We conclude that the MRP gene is transcribed in the nuclei of MEF cells but that the transcript either is not processed or is not stable in the cytoplasm. Hence the cells do not make the protein.



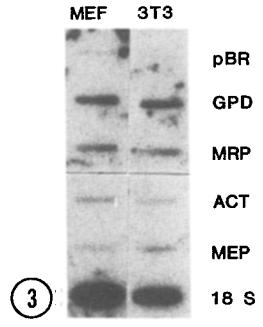


Fig. 2. Northern blot analysis of the MEP, MRP, and 2ar mRNA levels in mouse embryo fibroblasts at different passages. Ten µg of total cytoplasmic RNA was loaded in each lane, electrophoresed, blotted and probed as described in Materials and Methods. In the RNA extracted from cells at passages 11 and 12 the proportion of the total RNA that was represented by the three growth responsive genes was atypically low for unknown reasons.

Fig. 3. Nuclear run-on analysis of the transcriptional capacity of the nuclei of early passage mouse embryo fibroblasts and 3T3 cells. The slots contain 10 µg each of pBR322 or pBR322 containing the MRP clone; 1 µg of pBR322 bearing an insert corresponding to major excreted protein [MEP (6,14)], cytoplasmic actin (ACT) or glyceraldehyde phosphate dehydrogenase (GPD); and 0.4 µg of an 18s ribosomal RNA clone.

DISCUSSION

The MRP clone was isolated from a λ gt11 expression library of a cDNA prepared from mouse BNL cells (3). The conclusion that MRP and proliferin are the same is supported both by the identity between our partial sequence and the sequence of part of the longer proliferin sequence (3), and by the immunological relationship found by Nilsen-Hamilton et al. (16). Also, both cDNA clones (MRP and proliferin) identify a message of the same size (1 Kb) that is found almost exclusively in midgestational mouse placenta and is inducible in quiescent 3T3 cells by serum, fibroblast-derived growth factor, platelet-derived growth factor, or 12-O-tetradecanoylphorbol-13-acetate.

MRP/proliferin has not been shown to be a proto-oncogene, but given its relation to proteins with known growth stimulatory activities (prolactin, placental lactogen, growth hormone) it would be surprising if it were not capable of acting as an autocrine factor in certain situations. The autocrine

stimulation hypothesis states that cells can gain growth autonomy by any means that gives them the capability of producing, secreting, and responding to a growth factor that controls processes normally limiting in that cell type (17).

Alternatively, MRP/proliferin could influence the sensitivity of the immortal fibroblasts to another factor. For example, growth hormone and placental lactogen promote cell growth by increasing the secreted levels of IGF-1 and 2, which in rats are developmentally regulated; fetal fibroblasts produce and respond to IGF-2 only, whereas adult fibroblasts produce and respond to IGF-1 only (18,19). Also, it has been suggested that growth hormone promotes the differentiation of adipocytes from a pre-adipocyte mouse 3T3 cell line by clonal expansion, acting to elevate the sensitivity of the differentiating cells to IGF-1 and thereby to favour their outgrowth (20). Prolactin acts similarly in tissues that respond mitogenically (21). We are currently investigating whether MRP/proliferin has any effect on the proliferative ability 3T3 cells.

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