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Cell Biology

Transformation-dependent secretion of a low molecular weight protein by murine fibroblasts

(major excreted polypeptide/tumorigenicity/anchorage independence/radiolabeling/immunofluorescence)

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ABSTRACT **A protocol has been devised to radiolabel proteins secreted by murine fibroblasts *in vitro*. A radiolabeled polypeptide of molecular weight 35,000 is released into medium in relatively large amounts by transformed cells and in much smaller amounts by nontransformed fibroblasts. This major excreted polypeptide (MEP) is found in the medium of spontaneously transformed mouse cells and in the medium of mouse cells transformed by a DNA tumor virus, RNA tumor viruses, or methylcholanthrene. The appearance of MEP appears to be well correlated with anchorage independence in these transformed cells.**

MEP can be localized within the cytoplasm of transformed but not untransformed cells by indirect immunofluorescence. The presence of MEP within murine fibroblasts or in their culture medium serves as a novel biochemical marker of transformation. A biological role for this protein has not been assigned.

Malignant cells *in vivo* interact with each other, with neighboring host cells, and with the host immune system and blood supply. Some of this interaction is at the level of the cell surface, but much of it is postulated to occur through the release of specific humoral factors by tumor cells. Thus, using conditioned medium from transformed cells, investigators have found biological activities such as plasminogen activator (1), plasminogen-independent fibrinolysins (2), migration inhibitory factor (3), tumor angiogenesis factor (4), migration-stimulating activity (5), and multiplication-stimulating activity (6).

Studies of the proteins released by transformed cells have been hampered by the difficulty of obtaining large amounts of material free of contamination with serum proteins, cell proteins released by cell lysis, or proteins shed from the cell surface during handling of the cells. Consequently, a pulse–chase protocol was devised in which proteins destined for transport out of the cell could be radiolabeled and identified. By using this protocol, the major excreted polypeptides of transformed and untransformed mouse fibroblasts have been identified. The secretion of one major excreted polypeptide (MEP) of molecular weight 35,000 is markedly increased in a variety of transformed mouse fibroblasts. The presence of this polypeptide may serve as a biochemical marker of transformation.

MATERIALS AND METHODS

Cell Lines. Cell lines were maintained in Dulbecco–Vogt medium (National Institutes of Health Media Unit) supplemented with 10% calf serum (Colorado Serum Company) on Falcon tissue culture dishes at 37° in a 5% CO_2 atmosphere. Cells were trypsinized in 0.25% trypsin (Microbiological Associates) and were passaged using standard techniques. NIH Swiss 3T3 mouse fibroblasts (NIH) and their transformed derivative, Kirsten NIH (KNIH), were from C. Scher (Harvard Medical School). Medium from the nonproducer line KNIH was found to be free of viral particles and RNA-dependent DNA polymerase (reverse transcriptase) activity by E. Scolnick (National Cancer Institute). Abelson NIH and Harvey NIH were from E. Scolnick (National Cancer Institute). BALB/c 3T3 clone A31 and its derivatives Moloney BALB (Mol-BALB), SV-1 (a BALB/c 3T3 fibroblast transformed by simian virus 40 and passaged *in vivo*), methylcholanthrene-transformed BALB (Mc-BALB) and Swiss 3T3 were from the collection of I. Pastan (National Cancer Institute). Kirsten BALB (clone KA234) and a flat revertant of this strain, KA31-24 (7), were from C. Scher (Harvard Medical School). Primary mouse embryo fibroblasts were from B. Lovelace (National Cancer Institute). Spontaneous transformants of NIH (SP-NIH) and BALB/c 3T3 lines were obtained by cloning colonies that overgrew in the presence of 10% serum.

Viral Antigens and Antisera. Viral antigens and antisera were from J. Gruber, Viral Oncology Program, National Cancer Institute. Double immunodiffusion experiments (Hyland Ouchterlony plates, pattern D) were conducted between purified MEP from KNIH cells and goat antisera prepared against Tween/ether-disrupted Kirsten murine sarcoma virus, Gross leukemia virus, Moloney leukemia virus, and Rauscher leukemia virus, and against purified Gross leukemia virus p30 and Rauscher leukemia virus p30. Similar analysis was also made using rabbit antiserum against MEP versus purified Rauscher leukemia virus p30. The appearance of a precipitin band after 72 hr of incubation at 4° was considered a positive response.

Pulse–Chase Radiolabeling of Secreted Proteins. Freshly trypsinized cells were grown in medium with 10% serum for 48–72 hr until they reached a density of 1×10^6 cells per 60-mm Falcon tissue culture petri dish. The medium was then removed and the cells were carefully washed three times with 3 ml of medium lacking amino acids and serum. They were then incubated for 15 min with 3 ml of medium lacking amino acids and serum, but containing 10 μCi of mixed ^{14}C-labeled amino acids (New England Nuclear, NEC-445). This labeling medium was then discarded and the cells were washed two times with 3 ml of medium containing amino acids but lacking serum. The cells were then incubated for 3 hr in serum-free medium (a chase time sufficient to allow for secretion of proteins synthesized during the pulse period as determined by following release of trichloroacetic acid-precipitable radioactive material into the medium). The medium containing radiolabeled protein was collected and centrifuged at $500 \times g$ for 10 min to remove cell debris. Acid-precipitable radioactive material in the superna-

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Abbreviations: MEP, major excreted polypeptide; NIH, 3T3 NIH Swiss mouse fibroblasts; KNIH, Kirsten virus-transformed NIH; M_r, molecular weight; LETS protein, large external transformation-sensitive protein; CSP, cell surface protein.

tant was determined in a Beckman liquid scintillation counter using Econofluor scintillant (New England Nuclear) after collection on glass fiber filters. Acid-precipitable radiolabeled material in the cells was determined in the same manner after lysis with 0.5 M NaOH.

Polyacrylamide Gel Electrophoresis and Fluorography. Samples were concentrated for polyacrylamide gel electrophoresis by trichloroacetic acid precipitation. Two to five milliliters of medium was made 10% in trichloroacetic acid after addition of 20 μg of bovine serum albumin (Sigma) as carrier. After 30 min at room temperature, the samples were centrifuged at 12,000 × g for 30 min and the supernatants were discarded. After careful drainage and removal of excess liquid, the pellets were briefly washed with 1 ml of diethyl ether to remove trichloroacetic acid and dried with a stream of air. The pellets were redissolved in 30 μl of 0.15 M Tris·HCl, pH 6.8/10% (vol/vol) glycerol/1% sodium dodecyl sulfate/0.02 M EDTA/1% (vol/vol) 2-mercaptoethanol (sample buffer) and boiled for 2 min before application to the gels. A modification of the Studier (8) technique of polyacrylamide gel electrophoresis with a 5% stacking and 10% separating gel was used. The separating gel also contained 10% glycerol to enhance the sharpness and resolution of the protein bands (9). All electrophoresis reagents were from Bio-Rad. All gels were calibrated with the following molecular weight (M_r) standards: filamin, M_r 250,000; phosphorylase a, M_r 95,000 (Sigma); bovine serum albumin, M_r 68,000 (Sigma); pyruvate kinase, M_r 57,000 (Sigma); rabbit skeletal muscle actin, M_r 43,000; tropomyosin, M_r 33,000; and hemoglobin, M_r 16,000 (Sigma). Filamin, actin, and tropomyosin were from P. Davies (National Cancer Institute). Cell surface protein, M_r 220,000, was from K. Yamada (National Cancer Institute).

Fluorograms were prepared as described by Bonner and Laskey (10) and film images of the bands were quantitated as described by Laskey and Mills (11). Densitometry scans were made with a Joyce–Loebl densitometer and peak areas were determined using a Numonics electronic planimeter.

Preparation of Antibody to MEP. MEP was purified to apparent homogeneity from KNIH conditioned medium by Sephadex and DEAE-cellulose chromatography. Antisera to this purified antigen were prepared in two rabbits and gave single precipitin lines in double immunodiffusion experiments against crude or purified MEP preparations. Antisera from both rabbits specifically precipitated radiolabeled MEP from medium in which transformed cells had been grown. Details of the protein purification, the amino acid analysis, and the preparation and characterization of these antisera will be published elsewhere.

Indirect Immunofluorescence. Cells were grown for at least 48 hr after trypsinization in 30-mm Falcon tissue culture petri dishes, washed with Dulbecco's phosphate-buffered saline, fixed in 1% (wt/vol) formaldehyde in phosphate-buffered saline, and made permeable with 80% (vol/vol) acetone before treatment with rabbit antiserum to MEP and rhodamine-labeled goat anti-rabbit gamma globulin (Cappel). Acetone was omitted in experiments in which surface localization was examined. Rhodamine-labeled cells were observed and photographed with Polaroid type 107 film (60-sec exposure) on a Zeiss epifluorescence microscope.

RESULTS

Identification of major polypeptides secreted by transformed and untransformed fibroblasts

Analysis of the proteins secreted by mouse fibroblasts during the 3 hr following a 15-min pulse of ^{14}C-labeled amino acids reveals several major differences between transformed and untransformed cells. Fig. 1 compares an NIH Swiss 3T3 cell line with its RNA tumor virus (Kirsten virus)-transformed derivative. The banding patterns of the major radiolabeled polypeptides in the medium as shown by polyacrylamide gel electrophoresis in sodium dodecyl sulfate are relatively simple for both cell types (Fig. 1, lanes a and b), as compared to the patterns observed in the lysates of whole cells at the end of the 3-hr chase period (Fig. 1, lanes c and d). Furthermore, the medium from both transformed and untransformed cells is enriched for specific polypeptides that are not visible in the cell lysates at the end of the chase period, indicating that these are not present in the medium as a result of cell lysis. It is apparent that the untransformed cell line (NIH) releases high M_r polypeptides somewhat different from those released by the transformed derivative (KNIH), which also secretes a relatively large amount of a polypeptide with a M_r of approximately 35,000 (the major excreted protein, or MEP).

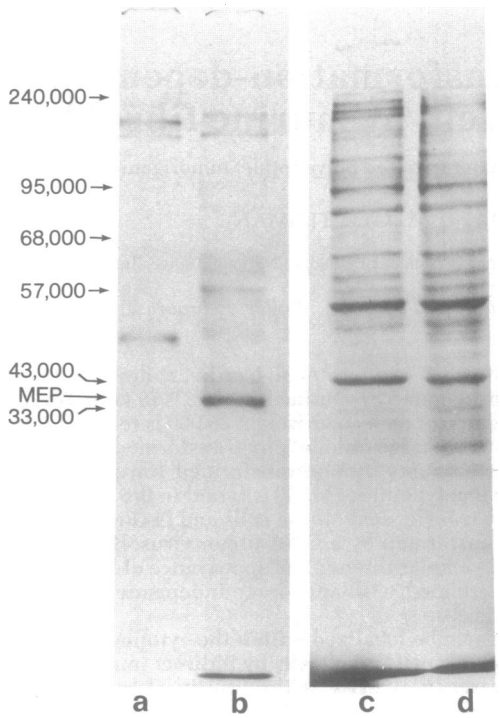

FIG. 1. Fluorography of radiolabeled polypeptides from NIH (untransformed) and KNIH (transformed) cells. Samples were prepared for electrophoresis as described in *Materials and Methods*. After removal of medium, cells were lysed with 2% sodium dodecyl sulfate, and aliquots of the lysates were boiled in sample buffer as described in *Materials and Methods*. Secreted proteins from NIH (lane a) and KNIH (lane b) and cell lysates from NIH (lane c) and KNIH (lane d) are shown. Approximately 6000 cpm were applied to a, 11,000 cpm to b, and 20,000 cpm to c and d, and the gels were exposed to the x-ray film for 72 hr. Proteins resulting from a small amount of cell lysis can be detected in this fluorogram because the gel was exposed to the x-ray film for a relatively long period of time.

The major bands from the untransformed cells shown in Fig. 1, lane a, are identified by the experiment shown in Fig. 2. Media from NIH and KNIH cells were incubated for various lengths of time in trypsin and collagenase and analyzed on a 7.5–15% exponential polyacrylamide gradient gel (12) to improve resolution of high molecular weight polypeptides. In the untransformed cell line (Fig. 2, lanes a–d), the highest molecular weight band in the medium has a mobility similar to that of a purified sample of large external transformation-sensitive

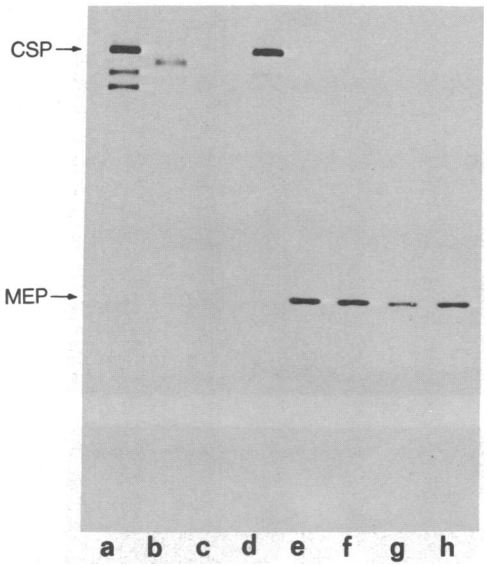

FIG. 2. Identification of the major radiolabeled polypeptides from NIH and KNIH cells. Cells were grown with 25 ml of medium in 150-mm Falcon plastic tissue culture petri dishes and labeled for 15 min with 50 μCi of ^{14}C-labeled amino acids. Aliquots (2 ml) of medium containing radiolabeled secreted proteins (15,000 cpm) were incubated at 37°. Medium from NIH (lane a) and KNIH (lane e) was incubated for 40 min with no additions; NIH (lane b) and KNIH (lane f) were incubated with 1 μg of trypsin (Microbiological Associates) for 5 min; and NIH (lane c) and KNIH (lane g) were incubated with 1 μg of trypsin for 40 min. Medium from NIH (lane d) and KNIH (lane h) was incubated with 2.8 μg of pure bacterial collagenase (gift of B. Peterkofsky, National Institutes of Health) for 40 min. Reactions were stopped by addition of 20 μg of carrier bovine serum albumin followed by 0.5 ml of 50% trichloroacetic acid, and the samples were processed as described in *Materials and Methods*. Fluorograms were made by exposure of the gels to x-ray film for 24 hr. The arrows show the positions of Coomassie blue-stained unlabeled CSP and MEP standards on the same gel.

protein (LETS protein) [also called cell surface protein (CSP)] from chick embryo fibroblasts (13, 14), and mild trypsin digestion produces a fragment of approximately 200,000-M_r characteristic of this molecule (14) (Fig. 2, lane b). The two next highest M_r bands are very trypsin sensitive and are also collagenase sensitive and have been tentatively identified on the basis of their M_r and this pattern of enzyme sensitivity as the procollagens, pro α_1 and pro α_2 (14, 15). In this experiment, where equal amounts of radiolabeled protein are applied to each lane in the gel, the transformed cell line (Fig. 2, lanes e–g) appears to secrete only a small amount of LETS (CSP), and very little collagen, but does produce a large amount of the 35,000-M_r polypeptide that is relatively trypsin resistant. Trypsin treatment of the high molecular weight proteins secreted by untransformed cells did not produce a stable fragment of 35,000 daltons.

The pattern of secretion of MEP appears to be relatively independent of the experimental conditions. It is not affected by changes in the pH of the medium from 6.5 to 8.0, by the state of growth of the cells, or by the nature of the radiolabel employed (data not shown). Release of MEP is also genetically stable, i.e., subclones of the transformed and untransformed cell lines have the same patterns of MEP secretion as their parent strains.

Correlation of the secretion of a 35,000 molecular weight polypeptide with transformation

In order to establish whether the secretion of the 35,000-M_r polypeptide (MEP) is related to the transformation process *per se*, a series of derivatives of a BALB/c 3T3 cell line were screened for secretion of this protein. Fig. 3 demonstrates that BALB/c strains transformed spontaneously or transformed by RNA tumor viruses (Kirsten and Moloney sarcoma viruses) (Fig. 3, lanes b and d), by a DNA tumor virus (simian virus 40) (Fig. 3, lane f) or by a chemical agent (methylcholanthrene) (Fig. 3,

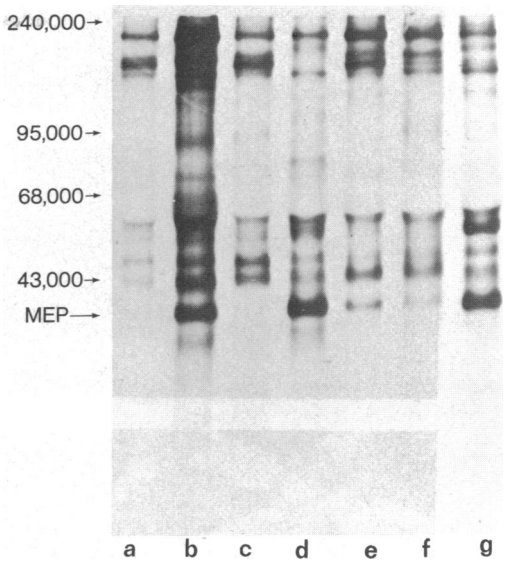

FIG. 3. Secretion of a 35,000-M_r polypeptide by various transformed mouse cells. Labeled media were prepared and subjected to electrophoresis; fluorograms were prepared. (Lane a) BALB/c, 10,000 cpm; (lane b) K-BALB/c, 30,000 cpm; (lane c) KA 234 (flat revertant of K-BALB), 10,000 cpm; (lane d) Mol-BALB, 12,000 cpm; (lane e) Mc-BALB, 9,000 cpm; (lane f) SV-1, 7,200 cpm; and (lane g) KNIH, 11,000 cpm.

Table 1. Correlation of MEP release with anchorage independence

Cell line	MEP (fraction of secreted protein)	Efficiency of cloning in 1.2% methylcellulose
	Low secretors	
NIH Swiss 3T3	<0.003	<0.005
Swiss 3T3	<0.004	<0.0001
BALB/c 3T3	0.002; 0.003*	<0.0001
BALB/c KA31-24	<0.004	<0.0001
Primary mouse embryo fibroblasts	0.009	NT
	Moderate secretors	
SP-NIH	0.029	1.2
SV-3T3	0.045	1.4
Mc-BALB	0.059	1.0
K-BALB	0.090	1.0
	High secretors	
Mol-BALB	0.29	1.0
Abel-NIH	0.30	NT
Ha-NIH	0.39	NT
K-NIH	0.28	0.9

MEP was quantitated as described in *Materials and Methods*. Anchorage independence was determined by plating efficiency in 1.2% methylcellulose (16) as compared to plating efficiency on Falcon tissue culture petri dishes in liquid medium. The cloning efficiencies of these cell lines ranged between 15 and 50%. NT, not tested.
* Secreted while cell growth was stimulated by 50% serum.

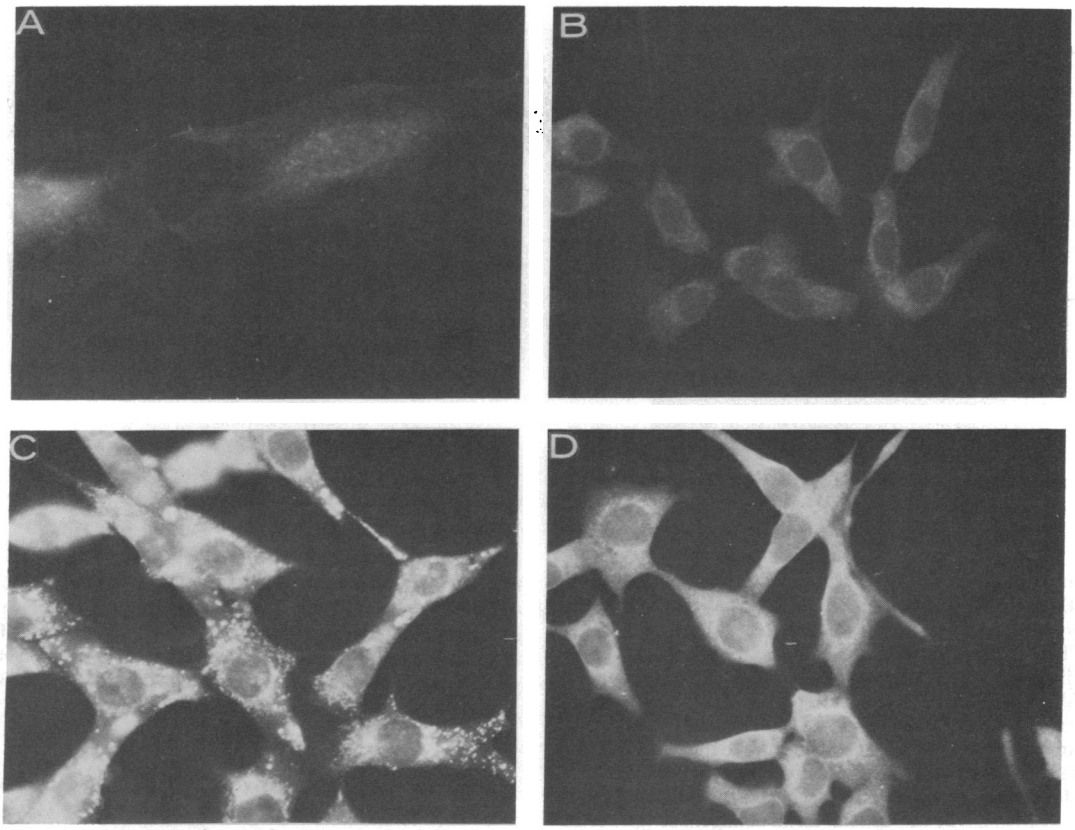

FIG. 4. Indirect immunofluorescence of NIH and KNIH cells. Cells were labeled and photographed as described in *Materials and Methods* with a 1/40 dilution of rabbit anti-MEP or preimmune serum followed by a 1/20 dilution of rhodamine-labeled goat anti-rabbit gamma globulin. (*A*) NIH (untransformed) rabbit anti-MEP preadsorbed with a 1/20 dilution of calf serum; (*B*) KNIH (transformed), preimmune serum; (*C*) KNIH, rabbit anti-MEP preadsorbed with a 1/20 dilution of calf serum; (*D*) KNIH, rabbit anti-MEP preadsorbed with MEP at 25 μg/ml. (×72.)

lane e) all excrete relatively large amounts of the 35,000-M_r polypeptide, whereas the parental cell line secretes barely detectable levels of this polypeptide under the conditions of this experiment. Furthermore, a flat revertant of the Kirsten virus-transformed derivative of BALB/c (Fig. 3, lane c) no longer secretes large amounts of the 35,000-M_r polypeptide.

The amount of MEP secreted by various mouse fibroblast cell lines is summarized in Table 1 and correlated with the anchorage independence of these cell lines. All of the cell lines examined secrete between 2% and 5% of the total protein they synthesize as measured by trichloroacetic acid-precipitable radiolabeled material. The untransformed cells secrete the smallest amount of the 35,000-M_r polypeptide (<1% of their total secreted protein), even when these cells are growing logarithmically or are stimulated by serum concentrations up to 50%. It is noteworthy that all untransformed mouse fibroblast cell lines examined do secrete a very small amount of this polypeptide.

Among the transformed cells, there are moderate and high secretors of MEP. In general, transformed derivatives of the NIH cell line appear to secrete more MEP than derivatives of other cell lines, and lines transformed by RNA tumor viruses secrete more MEP than cells transformed spontaneously, chemically, or by a DNA virus.

Immunofluorescence localization of MEP

By using the technique of indirect immunofluorescence, MEP was localized within transformed cells. Antisera from each of two rabbits showed very little localization on the surfaces of transformed (KNIH) and untransformed (NIH) cells (data not shown), confirming that MEP is not a cell surface protein. Preimmune sera from both rabbits did not localize intracellularly in any cell type examined (e.g., Fig. 4*B*), but both immune sera strongly labeled cytoplasmic granules in transformed cells (KNIH) (Fig. 4*C*), while barely labeling the cytoplasm of untransformed cells (Fig. 4*A*). Other transformed mouse cell lines (K-BALB, Mol-BALB, Mc-BALB, and SV-1) have also been shown to contain cytoplasmic granules that label with anti-MEP antiserum (data not shown). Preadsorption of the antiserum with purified MEP eliminated the granular cytoplasmic localization, (Fig. 4*D*), whereas preadsorption with calf serum did not interfere with cytoplasmic localization.

Antibodies prepared against purified Kirsten sarcoma virus, Gross leukemia virus, Moloney leukemia virus, Rauscher leukemia virus, and the p30 proteins from Gross and Rauscher leukemia virus did not produce precipitin bands when double immunodiffusion experiments were performed against purified MEP. Similarly, rabbit antiserum against MEP did not precipitate purified Rauscher leukemia virus p30, nor did this purified p30 comigrate with MEP on polyacrylamide gels.

DISCUSSION

By utilizing a protocol that radiolabels proteins secreted by cells in culture, the major excreted proteins from both transformed and untransformed cells have been characterized. It appears that untransformed mouse fibroblasts release mainly LETS protein (CSP) and the procollagens, pro α_1 and pro α_2, into the medium. On the other hand, transformed fibroblasts release smaller amounts of these proteins and secrete much larger

amounts of a 35,000-M_r protein (MEP). On the basis of mass, MEP represents approximately 1% of the total protein synthesized by one transformed cell line (KNIH), and approximately 30–40% of that cell's secreted protein. This is approximately the same as the amount of LETS protein (CSP) or total collagen secreted by the untransformed parent cell line. It has been previously observed that transformed mouse and chick cells synthesize decreased amounts of collagen (17, 18) and CSP (19). Adams *et al.* (20) concluded that the decrease in collagen and CSP biosynthesis in transformed chick cells was due to reduced levels of translatable mRNA for CSP and for collagen. The molecular basis for the increased MEP secretion seen after transformation has not yet been established.

The secretion of MEP appears to be transformation specific. This protein is released into the medium in large amounts (0.1 to 1% of total protein synthesized) by all of the transformed cells studied regardless of the means of transformation, and in much lower amounts (0.02% or less of total protein synthesis) by untransformed cells. The growth stimulation associated with transformation does not seem to be the mechanism responsible for increased release of MEP, because stimulation of the growth of cells obtained by plating at 20% of confluence or by growth in 50% serum results in only a slight increase in measurable MEP in the medium. Furthermore, complete inhibition of the growth of KNIH cells by sodium butyrate results in no decrease in the amount of MEP released by these cells (data not shown). It is noteworthy that primary mouse embryo fibroblasts, which have less density-dependent inhibition of growth than established 3T3 cell lines, make more MEP than the other nontransformed cells. Thus, in this case, MEP release correlates with degree of growth control.

MEP does not appear to be a product of an exogenous viral genome, because cells transformed chemically or spontaneously or by a variety of unrelated tumor viruses secrete it. It is also secreted to a small extent by nontransformed cell lines. In addition, it does not comigrate or crossreact with various murine sarcoma viral antigens, such as p30, and is secreted by non-virus-producing cell lines. These data do not rule out the possibility that MEP is the product of a previously undescribed endogenous viral genome.

It is unclear whether the release of MEP is a primary or early stage in the process of cell transformation, or whether it is a secondary effect. To date, it has not been possible to assign a biological role to MEP. Initial experiments indicate that the purified protein does not have protease activity, nor does it have growth-promoting or migration-stimulating activity. Its appearance in relatively large amounts suggests a stoichiometric or structural, rather than a catalytic, role for this protein.

Although the role of MEP in cell transformation is unclear at this time, the appearance of this protein in large amounts in cell culture medium might prove to be a useful marker of transformation. Because MEP release correlates with anchorage independence for the cell lines studied, and because anchorage independence has been well correlated for mouse fibroblasts with tumorigenicity *in vivo* (21), this easily detectable protein might prove to be a marker of tumorigenicity as well as of transformation.

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