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A novel regulatory mechanism for whey acidic protein gene expression

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When primary mouse mammary epithelial cells (PMME) are cultured on a basement membrane type matrix, they undergo extensive morphogenesis leading to the formation of 3-dimensional alveoli-like spherical structures surrounding a closed lumen. We show for the first time that cells cultured on basement membrane-type matrix express high levels of whey acidic protein (WAP) mRNA and secrete the protein into the lumen. The expression of WAP appears to be dependent upon the formation of the alveoli-like spheres: prevention of sphere formation by fixation or drying of the matrix abolishes the expression of WAP. Co-culturing PMME on native and fixed basement membrane matrix indicates that the suppression of WAP expression is dominant, thereby revealing the existence of a diffusible inhibitor(s). The inhibitory activity is present in the conditioned medium of PMME cultured on plastic surface and floating collagen gels, substrata that do not form alveoli and do not allow WAP expression. These findings are consistent with the model that the synthesis, or the action, of the WAP inhibitory factor is regulated by the tissue-like multicellular organization of mammary cells. When PMME do not have correct 3-dimensional structures, one (or more) inhibitor is secreted into the medium which suppresses WAP expression by an autocrine or paracrine mechanism. Nuclear run-on experiments suggest that the suppression of WAP expression is posttranscriptional. These results have obvious bearings on the understanding of the mechanisms by which cell-cell and cell-extracellular matrix interaction regulate tissue specific gene expressions.

Introduction

The influence of extracellular matrix (ECM)[1] on the expression of milk proteins in culture has been well documented by many laboratories including ours (Emerman *et al.*, 1977, 1979, 1981; Wicha *et al.*, 1982; Haeuptle *et al.*, 1983; Wilde *et al.*, 1984; Lee *et al.*, 1984, 1985; Rocha *et al.*, 1985; Li *et al.*, 1987; Chen and Bissell, 1987; Blum *et al.*, 1987). By using floating type I collagen gels (Emerman and Pitelka, 1977), and a basement membrane matrix, we and others have been able to express most of the skim milk proteins in culture. High levels of expression of caseins and transferrin genes and the secretion of these proteins on floating collagen gel and basement membrane matrix have been reported. While the level of mouse α-lactalbumin protein on floating gel is not very high, it is made in sufficient quantities to produce some lactose (Emerman *et al.*, 1981). Floating gel and a laminin substrata also allow high levels of α-lactalbumin expression in rat mammary cells (Wicha *et al.*, 1982). However, one of the major whey proteins in rodent milk, the whey acidic protein (WAP), is not expressed in cell culture, on floating gel, or on other culture conditions (Lee *et al.*, 1984, 1985; Rosen *et al.*, 1986). To our knowledge, none of the attempts to express high levels of WAP in cell culture has met with success.

WAP is expressed abundantly in the mammary gland of pregnant and lactating rodents (Piletz *et al.*, 1981; Henninghausen and Sippel, 1982; Hobbs *et al.*, 1982; Dandekar *et al.*, 1982; Pittius *et al.*, 1988b). The promoter sequence of the WAP gene and its binding protein have been studied in detail (Campbell *et al.*, 1984; Lubon and Henninghausen, 1987). In transgenic mice, WAP promoter-driven foreign genes have been shown to be expressed in tissue specific manner during lactation (Andres *et al.*, 1987; Schoenenberger *et al.*, 1988; Pittius *et al.*, 1988a). The uncoupling of WAP expression from other skim milk proteins and its failure to be expressed in cell culture point to a possibly unique regulatory mechanism for this protein. We therefore have been searching for additional factors that regulate WAP expression in vivo which may be "missing" in culture.

We have recently described a culture system for primary mouse mammary epithelial cells (PMME) which, in addition to high level expression of caseins and transferrin, is characterized by ex-

[1] Abbreviations: ECM, extracellular matrix; EHS, Engelbreth-Holm-Swarm tumor; PMME, primary mouse mammary epithelial cells; TGF-β, transforming growth factor-β; WAP, whey acidic protein.

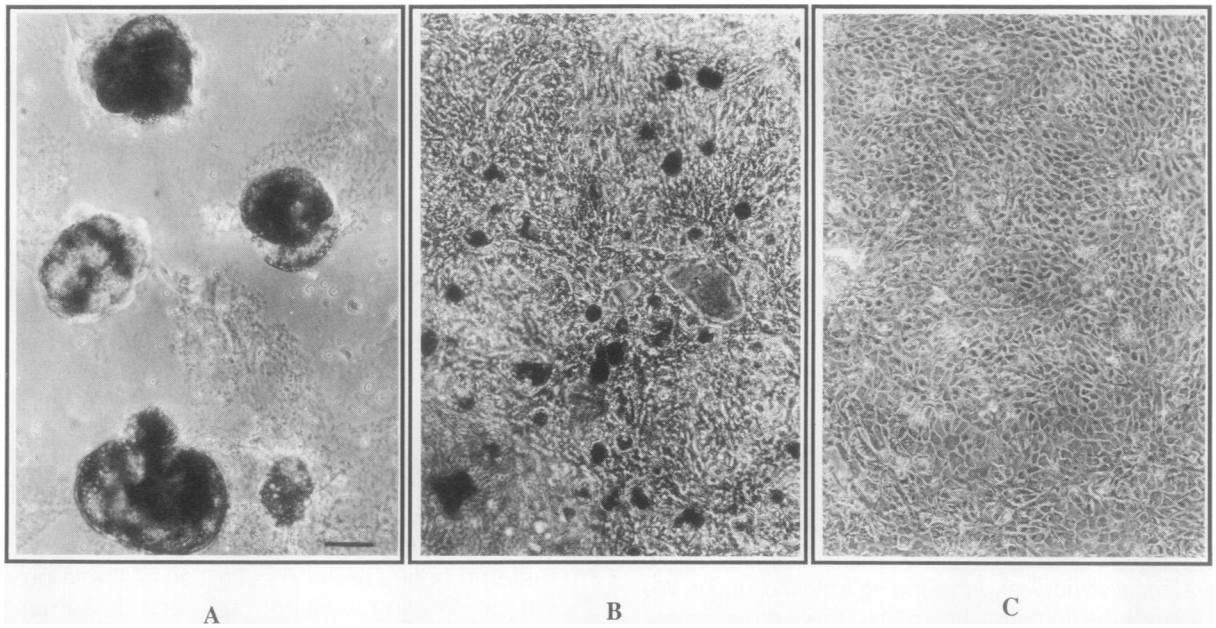

Figure 1. **Phenotype of cultured primary mammary epithelial cells.** Phase contrast photomicrographs of PMME cultured on EHS matrix (A), floating collagen gel (B) and plastic (C). Bar = 100 μm.

tensive morphogenesis (Barcellos-Hoff *et al.,* 1989). When plated on a matrix derived from Engelbreth-Holm-Swarm (EHS) mouse tumor (Kleinman *et al.,* 1986), PMME undergo a series of aggregation and reorganization leading to formation of 3-dimensional hollow spheres. Reminiscent of the secretory alveoli in vivo, the cells form tight junctions that give rise to a completely closed central lumen. Polarity is established not only morphologically, but also functionally: α-, β-, and γ-caseins are secreted vectorially into the lumen; transferrin and lactoferrin are secreted bidirectionally into the apical as well as the basal compartments.

We now have succeeded in producing cultures which are highly enriched for alveoli-like structures and report that under these conditions, WAP mRNA is expressed at high levels and the translated protein is secreted vectorially into the lumen. We further show that the expression of WAP appears to be dependent upon the formation of alveoli-like structures: the lack of expression in other culture conditions is due to the production of diffusible factor(s) that specifically suppress the expression of WAP mRNA.

Results

Expression of WAP by primary mouse mammary epithelial cells in culture

PMME cultured on EHS matrix formed 3-dimensional alveoli-like structures if the protein concentration of the EHS preparation was higher than 4 mg/ml, and cells were seeded at very high densities (>3 $\times$ 10^5/cm^2) (Figure 1A). Northern analysis of total cellular RNA from such cultures showed appreciable levels of the 0.65Kb WAP mRNA (Figure 2A, lane 1). In contrast, cells cultured on floating collagen gels (Figure 1B) or on plastic culture surface (Figure 1C) expressed little or no WAP mRNA (Figure 2A, lanes 2 and 3). The expression of transferrin mRNA, however, was high on floating collagen gels, as shown previously (Chen and Bissell, 1987; Figure 2A, bottom panel).

The synthesis and secretion of WAP protein was confirmed by western blotting analysis using a polyclonal anti-WAP antiserum. Proteins secreted by the alveoli-like spheres on EHS matrix can be divided into two fractions: basal (medium) and apical (luminal, EGTA extractable; Barcellos-Hoff *et al.,* 1989). WAP was detected only in the luminal fraction, indicating apical secretion; it was undetectable in the medium of cells cultured on floating gel or plastic (Figure 2B).

These results prompted further investigation of the role of EHS matrix and the formation of alveoli-like structures in allowing WAP expression.

Formation of 3-dimensional alveoli-like structures may be necessary for WAP expression

Two different, but related, mechanisms could underlie the expression of WAP on EHS matrix in

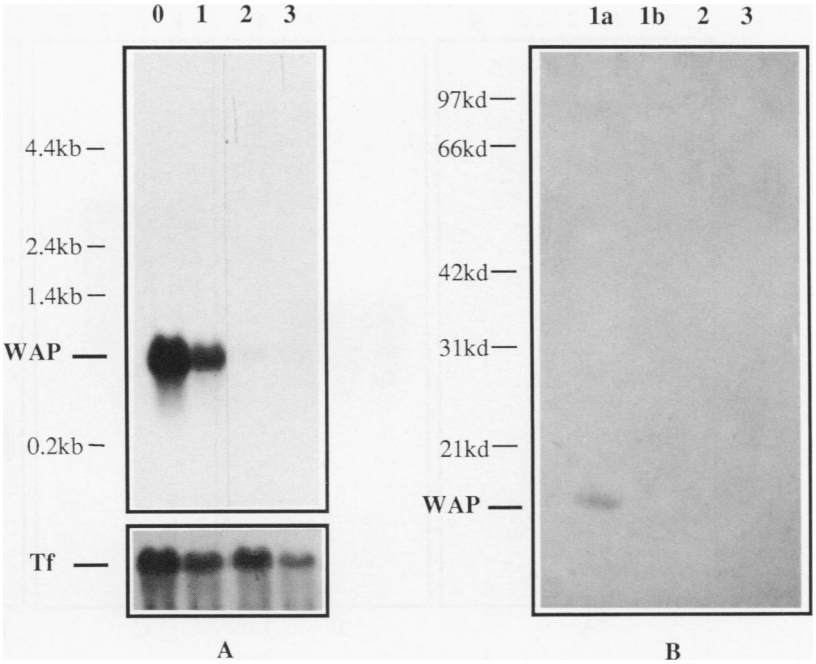

Figure 2. **Expression of WAP on EHS matrix.** (A) WAP mRNA expression in 11–14 d pregnant mammary gland tissue (lane 0) and PMME cultured on EHS matrix (lane 1), floating gel (lane 2) and plastic (lane 3). Total cellular RNA was isolated from the tissues and day six cultured cells. RNA (10 μg per lane) was resolved by 1.0% agarose gel and northern blots were hybridized to ^{32}P-labeled WAP cDNA (upper panel) and rehybridized to ^{32}P-labeled transferrin (Tf) cDNA (lower panel). (B) Secretion of WAP by PMME cultured on EHS matrix (EGTA fraction, lane 1a; medium fraction, lane 1b); on floating collagen gel (lane 2) and on plastic (lane 3). Equivalent volumes of media were loaded to each lane, separated by 6% SDS polyacrylamide gel, blotted onto nitrocelulose membrane, and reacted with an anti-WAP antiserum.

culture. Either the interaction of the cells with basement membrane molecules supplied by the EHS matrix is directly responsible, or alveoli-like sphere formation is additionally required. One can postulate that in the dynamic process of sphere formation on EHS matrix the flexibility of EHS may be necessary. To prevent flexibility, and hence sphere formation, 2% paraformaldehyde was used to fix the EHS coated on plastic dishes (see Methods). Cells cultured on fixed EHS matrix formed healthy monolayer; no spherical structures were developed, nor did clusters of aggregated cells remain at day 6 (Figure 3B). While the expression of transferrin and β-casein mRNA were only slightly reduced (Figure 4, B and C), the level of WAP mRNA of cells cultured on fixed EHS was reduced to <10% of the levels on native EHS matrix (Figure 4A, lane 2). To eliminate the possibility

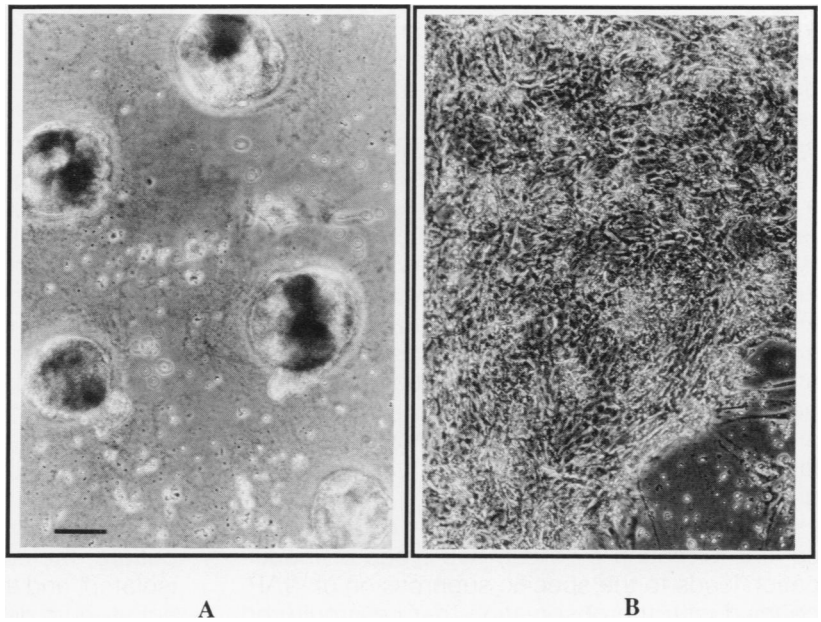

Figure 3. **Elimination of sphere formation on fixed EHS matrix.** Phase contrast micrographs of PMME cultured for 6 d on native (A) and fixed (B) EHS matrices. Bar = 100 μm.

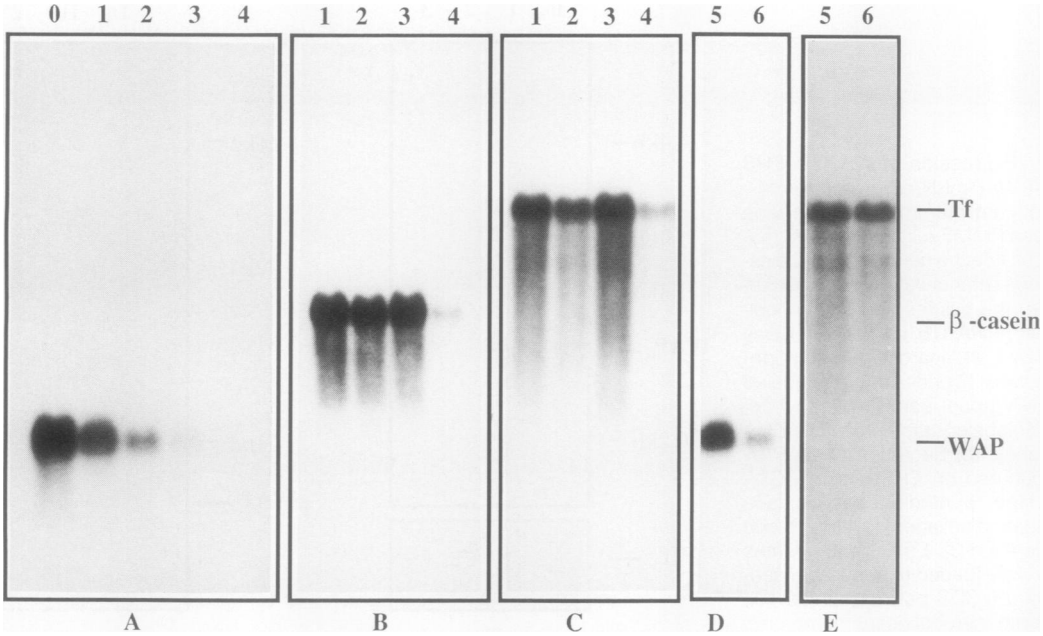

Figure 4. **Inhibition of WAP mRNA expression on modified EHS matrix.** Total cellular RNA was isolated from 11–14 d pregnant mammary gland tissue (lane 0), and PMME cultured on: EHS matrix (lanes 1 and 5); fixed EHS matrix (lane 2); air-dried EHS (lane 6); floating collagen gel (lane 3), and plastic (lane 4), electrophoressed (10 μg/lane) in 1.0% agarose gel, blotted and hybridized to ^{32}P-labeled WAP (A and D), β-casein (B) and transferrin (Tf) (C and E) cDNAs.

that native EHS contains additional factors that could specifically induce WAP expression but are inactivated during the chemical fixation of EHS, the EHS matrix was physically modified by allowing it to air-dry prior to cell plating. It has been reported previously that Sertoli cells grown on air-dried EHS film instead of a gelatinous EHS matrix form tight monolayers rather than aggregated clusters, without affecting the production of transferrin and androgen-binding protein (Janecki and Steinberger, 1987). PMME cultured on air-dried EHS substratum displayed a loose monolayer phenotype with various degrees of clustered cells remaining by day 6 (presumably due to rehydration of the gel). The expression of WAP by a monolayer-dominant culture was significantly reduced on air-dried EHS (Figure 4D). The reduction of expression was specific for WAP, as mRNA levels of transferrin and β-casein were unaffected (Figure 4E). This observation has been confirmed in other experiments where PMME were plated at a lower density, or on EHS preparations that had <4 mg/ml total protein. When the ratio of the area covered by alveoli-like structures to monolayer was <1:2, WAP expression was greatly reduced.

The finding that the prevention of alveoli-like sphere formation by these two types of modification leads to the specific suppression of WAP, coupled with the observation that cells cultured on floating collagen gels, while functional otherwise, do not express WAP, suggested that the organization into alveoli-like structures may be necessary for WAP expression. Due to the fact that formaldehyde fixation prevents sphere formation in a more consistent and controlled fashion than air-drying without apparent deleterious side effects (as shown by the morphology and the production of all the other milk proteins), this protocol was used in subsequent studies.

Factors released by PMME cultured on fixed EHS matrix suppress WAP expression

The following experiment was designed to test the possibility that soluble factors were involved in regulating the expression of WAP mRNA. A pair of glass coverslips coated with fixed or native EHS were placed in the same culture plate. PMME were seeded onto the coverslips, and the co-culture was carried for 6 d. The phenotype of the cells cultured on one coverslip was not affected by the presence of the other at the light microscopic level; the morphology of the alveoli-like spheres and the kinetics of their development on EHS in co-culture were indistinguishable from those on native EHS matrix alone. At day 6 of co-culture, the coverslips were separated, the RNA isolated, and the WAP mRNA expression on each substratum determined. The level of WAP mRNA

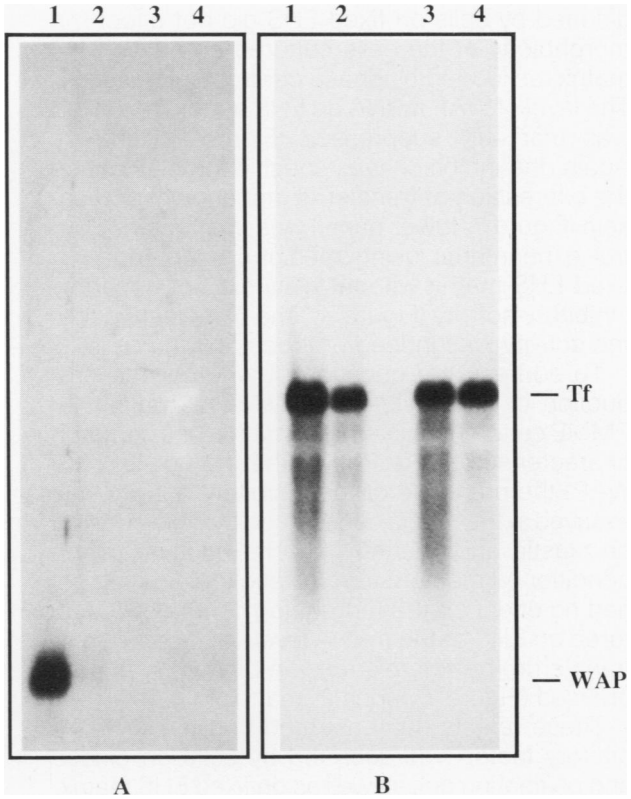

Figure 5. **Diffusible factors secreted by PMME cultured on fixed EHS matrix inhibit WAP mRNA expression in co-culture.** Total cellular RNA was isolated from PMME cultured on: EHS matrix (lane 1), EHS matrix co-cultured with fixed EHS (lane 2); fixed EHS matrix (lane 3); and fixed EHS co-cultured with EHS matrix (lane 4). RNA (10 μg) was loaded onto each lane and the northern blot hybridized with ^{32}P-labeled WAP cDNA (A), washed and rehybridized with ^{32}P-labeled transferrin (Tf) cDNA (B). In sister co-cultures, coverslips with identical coating had the same level of WAP and transferrin mRNA as on the respective substratum in the dish.

on fixed EHS was not affected by the co-culture and remained as low as cells cultured on fixed EHS alone (Figure 5A, lane 3). On native EHS matrix, however, the expression of WAP mRNA was markedly suppressed and its level was reduced to that observed on fixed EHS (Figure 5A, lane 2). The inhibition was essentially specific to WAP since the expression of transferrin and β-casein on the native EHS matrix in co-culture were only modestly reduced (Figure 5B, data for β-casein not shown).

Using this same system, the reversibility of the inhibition was determined. After 6 d of co-culture, the fixed EHS coverslips were removed and the cells on native EHS coverslips were cultured further. Three days after the removal of fixed EHS coverslips from the co-culture, WAP mRNA level on native EHS matrix was restored to the control level (Figure 6, lane 3).

These results imply that one (or more) diffusible factor(s) is released by mammary epithelial cells when cultured on fixed EHS matrix, leading to specific and reversible inhibition of WAP mRNA expression.

Inhibitor is present in conditioned medium of PMME cultures that do not reorganize into alveoli-like spheres

Having shown that diffusible inhibitory activity for WAP expression was present in the PMME cultured on fixed EHS matrix in co-culture, we then determined if this activity was detectable also in the medium conditioned by PMME cultured on fixed EHS matrix. The addition of medium con-

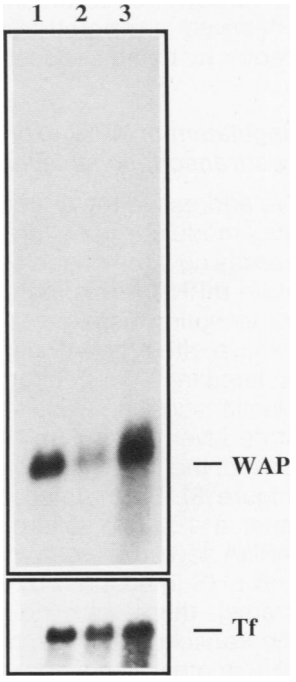

Figure 6. **Inhibition of WAP mRNA expression is reversible.** Total cellular RNA was isolated from PMME cultured on EHS matrix (lane 1); EHS matrix in co-culture with cells on fixed EHS for six days (lane 2); EHS matrix co-cultured with cells on fixed EHS for 6 d then removed from co-culture for 3 d (lane 3). 10 μg RNA/lane were analyzed by northern blotting.

ditioned by cells on fixed EHS did not affect the morphology of the cells cultured on native EHS matrix, as judged by phase contrast microscopy. The level of WAP mRNA on EHS matrix, however, was markedly suppressed (Figure 7, lane 4). Again, the inhibition was specific for WAP, since the expression of transferrin (not shown) or β-casein (Figure 7, lower panel) was unaffected. Control experiments using medium incubated with fixed EHS matrix without cells did not have any inhibitory activity (Figure 7, lane 5) indicating that the activity was indeed produced by the cells.

To address the question of whether the production of the inhibitory activity was unique to PMME cultured on fixed EHS matrix, or a common characteristic of the PMME that do not express WAP, the presence of the inhibitory activity was assayed in the media conditioned by cells cultured on plastic and floating gel. The addition of the conditioned medium from plastic and floating gel had no effect on the morphology of the cells cultured on EHS or the mRNA levels of β-casein and transferrin, but the expression of WAP was suppressed (Figure 7, lanes 2 and 3).

These results demonstrated that the WAP inhibitory factor was secreted by cells on plastic and on floating gel, as well as on fixed EHS matrix. Furthermore, since PMME cultured on plastic, floating gel, and fixed EHS share the common feature of not forming 3-dimensional spheres (Figures 1 and 3), and the medium conditioned by EHS cultures had little or no inhibitory activity, we concluded that the cells in the sphere either do not synthesize the inhibitor or the inhibitor is effectively prevented from acting on its own receptor by being sequestered in the lumen.

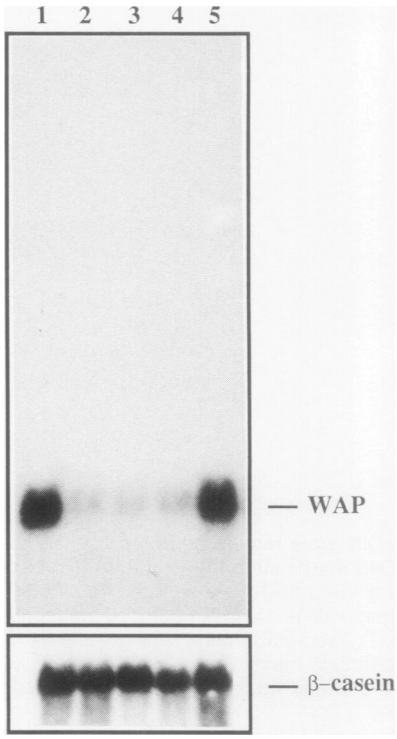

Figure 7. **Inhibition of WAP mRNA expression by conditioned media.** RNA was isolated from PMME cultured on EHS matrix in medium F12 (lane 1); conditioned media from cells cultured on plastic (lane 2); floating gel (lane 3); and fixed EHS (lane 4) and medium incubated with fixed EHS matrix without cells (lane 5). 10 μg RNA was electrophoressed in 1.0% agarose gel, the northern blots were hybridized with ^{32}P-labeled WAP cDNA and rehybridized with ^{32}P-labeled β-casein cDNA. Conditioned medium from cells cultured on EHS had no inhibitory effect.

Regulation of WAP expression is at the posttranscriptional level

We addressed the question of how the substrata may modulate the expression of WAP mRNA by assessing if the observed changes in the steady-state mRNA levels were transcriptional. Run-on transcription assay was used to determine the relative rates of WAP gene transcription in nuclei isolated from cells cultured on EHS matrix and on plastic surface. Despite the negligible steady-state level of WAP mRNA on cells cultured on plastic, the WAP gene was transcriptionally active (Figure 8). Furthermore, although there was at least a 100-fold difference in the steady-state mRNA level between cells cultured on plastic and EHS (measured by scanning the autoradiograms), there were no detectable increases in the transcriptional rate of WAP when cultured on EHS matrix within the sensitivity of this assay (Figure 8). In the same experiment, the relative rates of transcription for β-casein and transferrin on EHS matrix were increased two- and fivefold, respectively, in relation to cells on plastic.

Thus, maintenance of PMME on plastic results in the production of one or more factors that specifically alter the processing and/or stability of WAP mRNA.

Discussion

In this paper we have shown that the synthesis of WAP in cultures of midpregnant mammary epithelial cells is tightly coupled with the formation of 3-dimensional alveoli-like spheres aided by a reconstituted-basement membrane, the EHS matrix. When cells are maintained on plastic, floating collagen gels, fixed or air-dried EHS matrix, conditions that do not allow formation of these spheres, one or more diffusible inhibitors are secreted into the medium which specifically

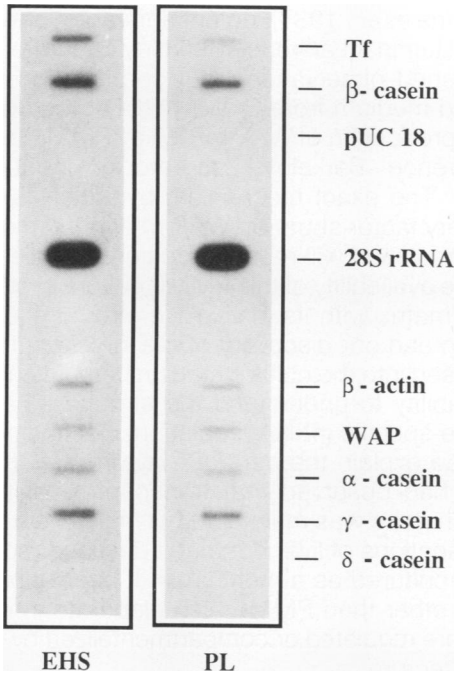

Figure 8. **Effects of EHS matrix on the relative rates of milk gene transcription.** Run-on assays were performed on nuclei isolated from PMME cultured on EHS matrix and on plastic as described in Methods. The ^{32}P-labeled nuclear RNA was hybridized to the indicated plasmid DNAs.

prevent the expression of WAP mRNA by an autocrine or paracrine mechanism.

The notion that tissue organization may be a prerequisite for expression of WAP germinated from the observation that only when alveoli-like structures were formed in culture was WAP mRNA detectable. Another indication of the association of WAP expression with sphere formation came from the kinetic study of WAP mRNA levels. WAP mRNA can be detected at day 3 of culture on EHS matrix with the level increasing through day 6 (data not shown). This coincides temporally with the formation of spheres and the development of the closed luminal inner space (Barcellos-Hoff *et al.,* 1989). Paraformaldehyde-fixed or air-dried EHS matrices allowed us to prevent sphere formation while still eliciting most of the differentiated functions of the cells as evidenced by the culture's ability to express β-casein and transferrin mRNA. These findings suggest that the interaction of the ECM molecules with their cellular receptors and the subsequent signal transmission that leads to a change in gene expression for transferrin and β-casein are not appreciably altered by the fixation or drying process. By prohibiting the formation of the alveoli-like structures, we therefore were able to separate the initial events of cell-matrix interactions from the process of sphere formation, allowing us to examine directly the consequences of the latter. The prevention of the spheres on EHS matrix blocked the expression of WAP, suggesting that formation of these structures is necessary for its expression. This finding solves a long-standing puzzle in our laboratory. Since we began to culture mammary cells on EHS matrix (Bissell *et al.,* 1985; Li *et al.,* 1987), we have observed sporadic expression of WAP. We now understand the reason behind those observations; we also can explain why WAP mRNA levels, unlike β-casein and transferrin, are not always as high as the levels found in vivo: it is technically difficult to produce cultures that are completely devoid of monolayers. The latter cells secrete the inhibitor into the medium, which not only shuts off WAP expression by the cells in the monolayer but would also lower WAP expression in alveoli-like structures. While it is possible that the observed inhibition on fixed EHS is a direct consequence of chemical modification or altered organization of the matrix itself, rather than the cellular architecture, the observations just described and the fact that prevention of sphere formation by air drying also specifically inhibit WAP expression argue against this possibility.

It is clear that what is required for WAP expression is a specific type of multicellular organization, since on floating gels clusters of cells are observed throughout the culture period, yet these cells are unable to support high levels of WAP expression. Our current results therefore imply the divergence of milk protein gene regulation at a new level: for genes such as transferrin and β-casein, a reconstituted-basement membrane is sufficient to sustain their expression in the presence of lactogenic hormones, while for WAP, another event, the further organization into a 3-dimensional configuration or sequestering the inhibitor, is necessary. The "missing factor" for WAP expression in culture therefore, is "correct" multicellular architecture.

The co-culture of cells on fixed and unfixed EHS matrix enabled us to explore the underlying mechanism of WAP expression. The dominance of the suppression, along with the fact that medium of cells on native EHS is devoid of the inhibitory activity, eliminate the need for postulating an additional positive signal, although does not rule it out completely. On the basis of the absence of WAP expression in WAP-transfected mammary-derived cell lines, Rosen and colleagues (1986) had postulated that WAP was probably under a negative regulation. Our data suggest that the alveoli-like structures formed on native EHS matrix

lead to WAP expression by inhibiting the inhibitor. The release of the inhibition could occur by one of the two mechanisms: either the cells in the sphere down-regulate the synthesis and the secretion of the inhibitor, or the inhibitor is secreted apically and is thus effectively prevented from acting on the basally located receptor due to the separation of the two compartments. Which of these two mechanisms is correct is under investigation.

The identity and the biochemical characterization of the inhibitor is of obvious interest. Our preliminary results show that exogenously added transforming growth factor-β_1 (TGF-β) specifically inhibits WAP expression without apparent gross morphological alterations of the alveoli-like structures. While it is tempting to postulate that the inhibitor may be a TGF-β-like molecule, it is possible that TGF-β may act by altering the ECM (Rizzino, 1988), hence altering gene expression (Bissell *et al.,* 1982). TGF-β has been shown to inhibit mouse mammary ductal growth but has little effect on the proliferation of the lobuloalveolar structures, and therefore was suggested to be a physiological regulator of mammary gland development (Silberstein and Daniel, 1987; Daniel *et al.,* 1989). Isolation and characterization of WAP inhibitory factor secreted on different substrata, as well as careful analysis of the level and the activity of TGF-β secreted by mammary cells, are necessary before additional conclusions can be drawn.

The possibility that WAP inhibitory factor may be involved in destabilizing or preventing correct processing of WAP mRNA is intriguing. The data presented here demonstrate that at least as judged by nuclear run-on assay, the dramatic difference between WAP mRNA on cells cultured on EHS and plastic is entirely post-transcriptional. Transferrin and β-casein on the other hand, showed both transcriptional and posttranscriptional regulation. The latter results agree with those reported by Rosen and his colleagues for these two milk proteins in COMMA-1-D cells (Eisenstein and Rosen, 1988; Rosen *et al.,* 1988). These investigators, however, could not find full-length WAP mRNA under any conditions (Eisenstein and Rosen, 1988), although a kinase-labeling procedure in which ^{32}P-labeled cellular RNA was hybridized to membrane-bound cDNA could pick up presumed degraded fragments of WAP mRNA on both plastic and floating gel. We do not detect any full-length WAP mRNA in COMMA-1D cells on EHS either (data not shown). Given our present knowledge, this is not surprising since COMMA-1D cells, despite a change in morphology and an increase in milk protein mRNA on EHS (Medina *et al.,* 1987), do not appear to form functional lumina (Lawrence Berkeley Laboratory, unpublished observation). Not surprisingly, conditioned medium from COMMA-1-D cells does inhibit production of WAP mRNA by PMME on EHS (Lawrence Berkeley Laboratory, unpublished data). The exact mechanism by which WAP inhibitory factor shuts off WAP mRNA synthesis or accumulation will require additional studies.

The availability of this PMME culture system on EHS matrix with its in vivo-like structural organization and our discovery of the inhibitory factors released into the culture medium should enhance our ability to understand the structural basis of tissue-specific mRNA regulation. Our finding may indeed explain the data of Clayton *et al.* (1985), who had observed that transcription of the albumin gene was maintained at high levels on tissue sections of liver but that it declined rapidly in cells cultured as a monolayer. It is possible that cells other than PMME also elaborate inhibitors that are regulated or compartmentalized by tissue architecture.

Methods

Cell and culture conditions

Epithelial cells were isolated from the mammary gland of 11- to 14-d-old pregnant CD-1 mice (Charles River, Wilmington, MA) by limited collagenase digestion and differential centrifugation as described (Lee *et al.,* 1985). Cells (9×10^6/60 mm dish) were seeded in F12 medium with 10% fetal calf serum, 50 μg/ml gentamycin, and lactogenic hormones, insulin (bovine pancreas, 5 μg/ml, Sigma, St. Louis, MO), hydrocortisone (1 μg/ml, Sigma), and prolactin (bovine, 3 μg/ml, obtained from National Hormone and Pituitary Program, contracted to NIADDK, Baltimore, MD) and incubated at 37°C in 5% CO_2. Serum was removed from the culture 48 h after plating and medium was changed daily thereafter. Rat tail collagen was prepared as described (Emerman and Pitelka, 1977), laid onto the culture dish at 3 ml/60 mm dish and detached to float 48 h after plating. EHS matrix was extracted from the EHS tumor by established procedures (Kleinman *et al.,* 1986). The matrix was coated onto the culture dish at 250 μl/60 mm dish and preincubated for 3–4 h in a humidified incubator at 37°C before plating to allow the matrix to gel as described (Li *et al.,* 1987; Barcellos-Hoff *et al.,* 1989). To fix the matrix, the plates coated with EHS were incubated with 2% paraformaldehyde (pH 7.0) for 1 h followed by overnight wash with several changes of PBS containing 0.1% glycine, and equilibrated with culture medium for plating. Conditioned medium was collected every 24 h from the donor culture, supplemented with fresh hormones, and passed through 0.45 μm filter prior to addition to the recipient cultures.

RNA analysis

Total cellular RNA was isolated by the guanidine isothiocyanate method (Chergwin *et al.,* 1979). The RNA was electrophoressed in 1.0% agarose containing 1.6 M formaldehyde in MOPS buffer (0.2 M morpholinepropanesulfonic acid, 50 mM sodium acetate, 5.0 mM EDTA pH 7.0). RNA was transferred to Hybond membrane (Amersham Corporation, Arlington

Heights, IL) by the established procedure (Maniatis *et al.,* 1982). The prehybridization and the hybridization were carried out as described (Chen and Bissell, 1987). The probes were labeled to 1–10 × 10^8 cmp/μg DNA by nick translation or primer extension, and added at 1 × 10^6 cpm/ml of the hybridization solution. The cDNA clones of caseins were gifts from Jeff Rosen (Baylor College of Medicine, Houston, TX), WAP cDNA was a gift from Lothar Henninghausen (National Institutes of Health, Bethesda, MD), and the transferrin clone was isolated in our laboratory (Chen and Bissell, 1987).

Western blotting

Immunoblotting of WAP was performed as described (Lee *et al.,* 1984), using an anti-mouse WAP antisera (Piletz *et al.,* 1981).

In vitro transcription assay

Cells cultured on plastic and EHS matrix were harvested by trypsinization. The cells were incubated on ice for 10 min in swelling buffer containing 0.1 M hexelene glycol, 1 mM KCl, 0.6 mM PIPES pH 6.8. Triton X-100 was then added to 0.1% and KCl to 140 mM followed by homogenization by Dounce homogenizer (B pestle) and centrifuged at 800 × *g* for 5 min. The nuclei were washed twice in cold reaction buffer containing 10 mM TrisHCl pH 8.1, 20% glycerol, 140 mM KCl, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 14 mM β-mercaptolethanol. The incorporation of ^{32}P-UTP into the nuclei was carried out in the same reaction buffer plus 0.5 mCi of α^{32}P-UTP (3000 Ci/mmol, Amersham Corporation). After 20 min, the nuclei were pelleted and resuspended in 0.5 M NaCl, 10 mM Tris-HCl pH 7.4, 50 mM $MgCl_2$ with 100 U/ml DNase1, 0.4 mg/ml protein kinase K, 10 mM EDTA, and 0.5% SDS for 30 min at 37°C. Labeled RNA was obtained by phenol extraction followed by ethanol precipitation. For hybridization, denatured, linearized cDNA (5 μg) was slot blotted onto nitrocellulose membranes and immobilized by baking at 80°C for 2 h. Conditions for prehybridization and hybridization were the same as for northern blotting. Blots were hybridized with 10 × 10^6 cpm labeled RNA for 40 h. The β-actin cDNA clone and genomic clone to 28 S cDNA were gifts from Marc Kirschner (University of California, San Francisco) and James Sylvester (University of Pennsylvania), respectively.

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