MOLECULAR AND CELLULAR BIOLOGY, Aug. 1988, p. 3183–3190
0270-7306/88/083183-08$02.00/0

Vol. 8, No. 8

Both Cell Substratum Regulation and Hormonal Regulation of Milk Protein Gene Expression Are Exerted Primarily at the Posttranscriptional Level

RICHARD S. EISENSTEIN† AND JEFFREY M. ROSEN*

Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

Received 25 February 1988/Accepted 16 May 1988

The mechanism by which individual peptide and steroid hormones and cell-substratum interactions regulate milk protein gene expression has been studied in the COMMA-D mammary epithelial cell line. In the presence of insulin, hydrocortisone, and prolactin, growth of COMMA-D cells on floating collagen gels in comparison with that on a plastic substratum resulted in a 2.5- to 3-fold increase in the relative rate of β-casein gene transcription but a 37-fold increase in β-casein mRNA accumulation. In contrast, whey acidic protein gene transcription was constitutive in COMMA-D cells grown on either substratum, but its mRNA was unstable and little intact mature mRNA was detected. Culturing COMMA-D cells on collagen also promoted increased expression of other genes expressed in differentiated mammary epithelial cells, including those encoding α- and γ-casein, transferrin, malic enzyme, and phosphoenolpyruvate carboxykinase but decreased the expression of actin and histone genes. Using COMMA-D cells, we defined further the role of individual hormones in influencing β-casein gene transcription. With insulin alone, a basal level of β-casein gene transcription was detected in COMMA-D cells grown on floating collagen gels. Addition of prolactin but not hydrocortisone resulted in a 2.5- to 3.0-fold increase in β-casein gene transcription, but both hormones were required to elicit the maximal 73-fold induction in mRNA accumulation. This posttranscriptional effect of hormones on casein mRNA accumulation preceded any detectable changes in the relative rate of transcription. Thus, regulation by both hormones and cell substratum of casein gene expression is exerted primarily at the post transcriptional level.

The expression of the milk protein genes in mammary epithelial cells (MEC) is regulated by a variety of factors, including peptide and steroid hormones and cell-cell and cell-substratum interactions (6, 19, 26, 32, 34, 53). Much of the work directed at defining the hormonal requirements for milk protein mRNA and protein accumulation has been done with mammary gland explant cultures. These studies have demonstrated that hydrocortisone and prolactin in the presence of insulin stimulate casein gene expression at the transcriptional and post transcriptional levels (24, 28). Due to difficulties inherent in the explant system, it has not been possible to define precisely at the molecular level the separate roles of individual peptide and steroid hormones in regulating casein gene expression.

Primary cultures of MEC provide an alternative to mammary explant cultures, although they are still subject to problems of biological variability and cell recoveries. However, primary MEC cultured on a plastic substratum dedifferentiate and only express very low levels of casein mRNA and protein (15, 35). Emerman and Pitelka (15) were able to maintain MEC differentiation through use of floating, hydrated, type I collagen gels. More recently, cell-substratum and cell-cell interactions have been shown to affect milk protein gene expression at several levels, including mRNA accumulation and milk protein stability and secretion, in addition to influencing other aspects of MEC physiology (4, 6, 16, 32, 34, 35, 40, 45, 49, 52). The mechanisms by which cell-substratum interactions affect any of these processes in MEC are not yet understood.

Development of a normal MEC line, COMMA-D, has overcome many of the problems inherent in both explant and primary cultures of MEC (12). COMMA-D is a mixture of several cell types with approximately 10 to 20% of the cells capable of casein synthesis (40). Furthermore, casein mRNA accumulation in these cells is regulated by both hormones and cell-substratum interactions (40). Because it is possible to obtain large amounts of cells grown under defined hormonal conditions, we were able to define for the first time the individual roles of prolactin and glucocorticoids in the regulation of casein gene transcription and mRNA accumulation and the temporal relationship between these two processes. In addition, we studied the effects of cell-substratum interactions on casein gene transcription and mRNA accumulation. These studies demonstrate that, like the effect of prolactin and hydrocortisone on casein mRNA accumulation, the cell-substratum regulation of casein gene expression is exerted primarily at the post transcriptional level.

MATERIALS AND METHODS

Cell culture. COMMA-D cells of passage 10 or lower were grown in Dulbecco modified Eagle medium supplemented with glutamine, 5% fetal calf serum (FCS), insulin (5 μg/ml; Sigma), and gentamicin sulfate (50 μg/ml). For the cell-substratum experiments, cells were plated onto either plastic dishes or type I collagen gels (4). After 2 to 3 days of culture, the gels were released and allowed to float in the medium. All hormone experiments were performed with cells grown on floating gels. For these experiments, cells were plated onto gels and allowed to grow for 1 day before the medium was changed to one containing 10% charcoal-dextran-

* Corresponding author.
† Present address: Department of Applied Biological Sciences, Building 56-224, Massachusetts Institute of Technology; Cambridge, MA 02139.

stripped horse serum instead of FCS. After 48 h the medium was changed and the gels were floated. After 24 h, the medium was changed to either insulin (I) alone, I plus hydrocortisone (F) (1 μg/ml; Sigma) (IF), I plus prolactin (M) (1 μg ml; National Hormone and Pituitary Program, National Institutes of Health, Bethesda, Md.) (IM), or I, F, and M (IFM).

In vitro transcription assay. Cells grown on plastic (four 100-mm dishes; 1×10^7 to 2×10^7 cells per dish) were harvested with 1 ml of 10× trypsin-EDTA (GIBCO) for 3 to 5 min at room temperature. Then, 10 ml of Hanks balanced salt solution (HBSS) and 100 μg of soybean trypsin inhibitor (SBTI; Sigma) were added, and the cells were pelleted at 500 × *g* for 5 min.

Collagen gels were digested as described elsewhere (4). Cells were pelleted and suspended in 3 ml of 1× trypsin-EDTA and left on ice, with occasional mixing, for 5 min. Trypsinization was necessary for isolation of adequate quantities of nuclei. After addition of 10 ml of HBSS and SBTI to 10 μg/ml, the cells were pelleted as described above.

Cell pellets were resuspended in 4 ml of 0.3 M sucrose–25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 8.0)–3 mM $MgCl_2$–0.25 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid]–0.5 mM dithiothreitol (DTT)–0.1% Nonidet P-40–25 μg SBTI per ml–10 μg of leupeptin and chymostatin per ml and homogenized in a Dounce homogenizer (B pestle) and centrifuged at 500 × *g* for 5 min. The nuclear pellet was frozen at −70°C as described (38). No difference in total or specific transcription was observed between fresh nuclei and nuclei frozen for up to 2 weeks.

Nuclei were thawed briefly and centrifuged at 500 × *g* for 5 min and suspended in 75 μl of buffer T (150 mM potassium acetate, 5 mM $MgCl_2$, 1 mM DTT, 50 mM HEPES [pH 8.0], 1 mM $MnCl_2$, 10% glycerol), and 100 μl of this preparation was added to 150 μl of buffer T containing 10.0 mM phosphoenolpyruvate, 125 μg of pyruvate kinase per ml, 0.5 mM each ATP, CTP, and GTP, and 1 mCi of [α-^{32}P]UTP (3,000 Ci/mmol; Amersham). After 20 min the nuclei were pelleted and suspended in 200 μl of HBSS. RNA was obtained as described (9).

The [^{32}P]RNA was nicked with 0.1 N NaOH as described (20). Usually, 30×10^6 to 40×10^6 cpm were hybridized, in 300 μl, to filter-bound cDNAs. DNAs (5 μg per spot) were bound to nitrocellulose after denaturation in 0.1 N ammonium hydroxide at 100°C for 10 min and cooling on ice for 10 min. Ice-cold 2 M ammonium acetate was added to bring the DNA concentration to 25 μg/ml, and 200 μl was applied to each sample well. Filters were baked at 80°C in vacuo for 2 h. Hybridization reactions were done in silanized 5-ml polypropylene tubes immersed in a gyrorotatory water bath set at 45°C. Prehybridization was for 4 h as described (38) with poly(A) (100 μg/ml), yeast RNA (50 μg/ml), 0.1 mM UTP, and 0.1% (wt/vol) sodium pp_i. This buffer was replaced with the same buffer plus [^{32}P]RNA and 2,500 cpm of [^{3}H]RNA as an internal standard. Hybridization reactions, in triplicate, were done for 72 h. Filters were washed as described (38) except that after treatment with RNases A and T_1 (100 U of T_1), they were washed in buffer A (38) plus proteinase K (100 μg/ml) and 0.2% sodium dodecyl sulfate (SDS) for 30 min, during which time the temperature was increased to 55°C, and finally in buffer A plus 0.1% SDS at 65°C for 1 h. [^{32}P]RNA was eluted from dried filters as described (38). The counts hybridized were determined by liquid scintillation counting. Nonspecific hybridization to vector (pBR322) sequences was less than 1 ppm (cpm bound per 10^6 input cpm). Hybridization efficiency, determined by RNA internal standards, was 10% for β-casein, 20 to 30% for α-casein and actin, and 40 to 50% for histone H4. Signal-to-noise ratios for β-casein were at least 4:1. RNA internal standards were transcribed from cDNAs cloned into SP64 or pGEM4 (Promega) by using SP6 or T7 polymerase in the presence of 0.5 mM of each nucleoside triphosphate and 100 μCi of [5,6-^{3}H]UTP (ICN). Full-length RNAs were obtained after electrophoresis through 5% acrylamide–8 M urea gels.

Under the conditions described for the transcription assay, incorporation of [^{3}H] UTP was linearly related to the input of nuclei over a range of 0.8×10^7 to 8×10^7 nuclei per ml. UTP incorporation was linear for 20 min, and 1.0×10^8 to 1.5×10^8 cpm of [^{32}P]RNA was usually recovered. Strand specificity of transcription was determined by hybridizing [^{32}P]RNA to both orientations of β-casein cDNA cloned into M13. Hybridization of [^{32}P]RNA was detected only to the coding strand of β-casein DNA sequences. Similar results were obtained from cells exposed to I or IF alone, but the signal intensity was decreased. Since transcription was strand specific and hybridization efficiency was higher when a double-stranded plasmid was used (10 to 15%) than when M13 DNA was used (5% efficiency), the former was used for the studies described in the Results section. The counts hybridized to α- and β-casein, actin, and histone H4 cDNAs were directly proportional to the input [^{32}P]RNA. Transcription of the α- and β-casein and actin genes was inhibited 85% by a concentration (1 μg/ml) of α-amanitin known to specifically inhibit RNA polymerase II (not shown) (48).

DNA clones. Mouse casein and whey acidic protein cDNAs (7, 23) were cloned into SP64 or M13mp8. The following additional clones were used: histone H4 (22), transferrin (8), malic enzyme (1), phosphoenolpyruvate carboxykinase (PEPCK) (2), and nonmuscle type 5 actin (3).

RNA isolation and analysis. RNA was isolated as described (9). RNA blotting, on nylon membranes (Pall Biodyne), was done with glyoxal (39). RNA probes were synthesized as described before (41). Kinase labeling of total RNA was done as described elsewhere (47) except that the [^{32}P] RNA was treated with proteinase K and extracted with phenol and $CHCl_3$ before use. Hybridization and washing of filters were performed as described above, including the use of RNA internal standards.

RESULTS AND DISCUSSION

Sufficient amounts of [^{32}P]RNA (1.0×10^8 to 1.5×10^8 cpm) were recovered under the conditions used in our transcription assays to generate the necessary hybridization inputs required to accurately quantitate specific transcription rates in uninduced cells (grown on plastic with I or IFM present). The transcription assay was also optimized to ensure that the signals detected were significantly (by at least 4 to 1) greater than background, strand specific, derived from the linear portion of the assay, and the result of RNA polymerase II transcription (see Materials and Methods). In all transcription assays, RNA internal standards were included to determine the hybridization efficiency, and transcription of control genes was examined to determine the specificity of the response observed. Once the fidelity and specificity of the assay were established, it was possible to examine the effects of both cell-substratum interactions and hormones on COMMA-D cell gene expression.

Effects of cell substratum on gene expression in COMMA-D cells. The substratum on which COMMA-D cells were grown had a major effect on expression of several milk protein

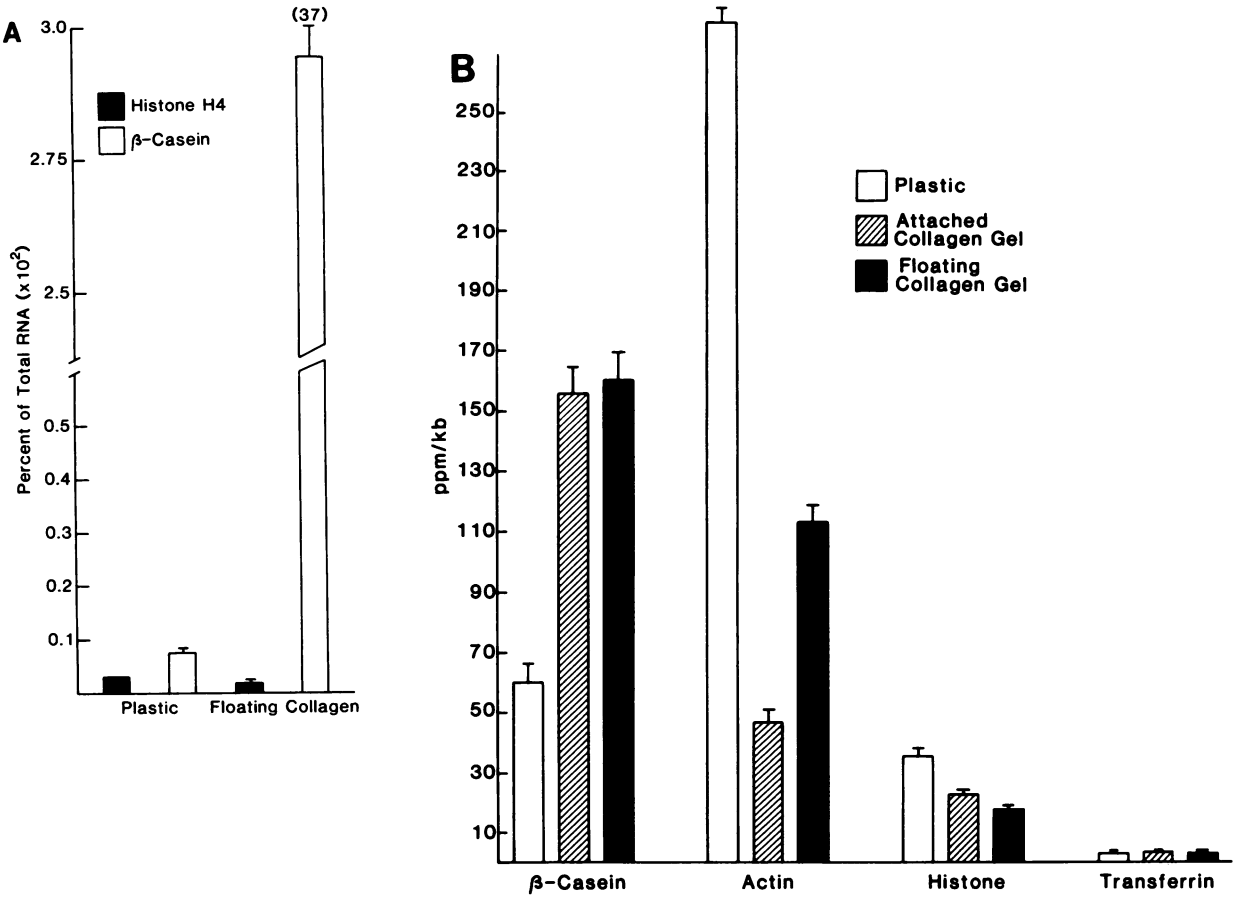

FIG. 1. Cell substratum induction of β-casein mRNA in COMMA-D cells. COMMA-D cells were grown on plastic or floating collagen gels in the presence of IFM for 48 h as described in Materials and Methods. Total RNA (5 μg) was kinase labeled, and 40 × 10^6 cpm was hybridized to immobilized cDNAs. The number above the β-casein bar refers to the fold induction compared with the level measured in cells grown on plastic. Values are expressed as mean ± standard error of the mean (SEM) for triplicate hybridizations. (B) Effect of cell substratum on specific gene transcription. COMMA-D cells were grown on plastic or attached or floating collagen gel substrata with IFM present for 48 h. Runoff transcription was performed as described in the text. The ^{32}P-labeled transcripts (30 × 10^6 cpm) were hybridized to immobilized cDNAs. The relative rate of transcription was calculated as follows: ppm/kb = [(cpm bound to cDNA − cpm bound to pBR322) × (100/% efficiency) × (1/input cpm [in 10^6 cpm]) × (1/insert size [kb])]. Values are expressed as mean ± SEM for triplicate hybridizations.

genes. Cells grown on plastic in the presence of IFM expressed a low level of β-casein mRNA, approximately equivalent to that observed for histone H4 mRNA (Fig. 1A). In cells grown on collagen gels, β-casein mRNA accumulated to higher levels (Fig. 1A). Culturing cells on attached collagen gels increased the β-casein mRNA concentration by 20-fold (not shown), whereas allowing the gel to float in the medium resulted in a 37-fold induction of β-casein mRNA. Histone mRNA decreased in abundance under these conditions.

The relative rate of β-casein gene transcription was about 60 ppm in cells grown on plastic and increased to 150 ppm when cells were grown on collagen (Fig. 1B). This 2.5-fold effect of cell substratum on β-casein gene transcription was not sufficient to account for the much larger 37-fold increase in β-casein mRNA accumulation. Therefore, the cell substratum regulates β-casein mRNA accumulation mainly by modulating RNA stability. The increased amount of β-casein mRNA observed once the collagen gel was floated occurred without any further change in transcription.

The substratum on which the cells were grown also affected actin and histone gene transcription. Compared with cells grown on plastic, actin gene transcription decreased by 80 and 60% from that in cells grown on attached and floating collagen gels, respectively (Fig. 1B). Histone gene transcription decreased by 30% for cells grown on attached collagen and 50% for cells grown on floating collagen gels compared with the rate observed in cells grown on plastic. The histone H4 mRNA level decreased by a similar magnitude (Fig. 1A).

We also examined expression of another milk protein gene, the WAP gene. In COMMA-D cells, intact WAP mRNA was detectable at a level less that 0.1% of that observed in lactating mammary tissue (not shown). This contrasted sharply with β-casein mRNA, which was present in COMMA-D cells at a level between 10 and 20% of that found in RNA from lactating tissue (J. M. Rosen, R. S. Eisenstein, A. R. Schlein, P. Poyet, K. F. Lee, and L.-Y. Yu-Lee, *in Proceedings of the Third International Congress on Hormones and Cancer*, in press). However, with kinase-labeled RNA and runoff transcription assays, substantial amounts of WAP RNA transcripts were detectable irrespective of the substratum (Fig. 2). These results, although in contrast to those determined by Northern (RNA) blotting, were not an artifact of the kinase-labeled RNA procedure, as both of these methods gave similar results for α- and β-casein, actin, and histone H4 mRNAs (not shown). The

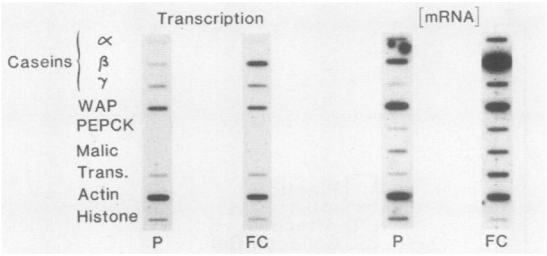

FIG. 2. Effects of different substrata on specific gene transcription and mRNA accumulation in COMMA-D cells. Cells were grown on plastic (P) or floating collagen gels (FC) harvested as described in the text. Nuclei were isolated for in vitro transcription, or total RNA was isolated for measurement of mRNA accumulation as described in the legend to Fig. 1. Trans., Transferrin.

WAP gene was transcribed at a rate similar to that observed for β-casein in cells grown on floating collagen gels (Fig. 2). Thus, the WAP gene was transcribed constitutively, but WAP mRNA was unstable. RNA blotting detected a low level of intact WAP mRNA, while the kinase-treated RNA approach apparently detected degraded fragments of WAP mRNA that were not detectable by blotting.

Cell substratum affected the expression of other genes that, like β-casein, are characteristically expressed to a greater extent as the mammary gland becomes more differentiated (8, 14, 26, 36, 37). The mRNAs for α- and γ-casein, PEPCK, malic enzyme, and transferrin were more abundant in COMMA-D cells grown on floating gels (Fig. 2). In contrast, actin and histone H4 mRNA levels were slightly decreased in cells grown on floating collagen gels. Transcription of α- and γ-casein and transferrin genes (Fig. 1B and 2) was increased minimally, if at all, again suggesting that the effects of cell substratum in promoting differentiation of COMMA-D cells depend to a large extent on regulation of gene expression at the posttranscriptional level.

These results support the concept that posttranscriptional processes are an important mechanism by which the cell substratum affects milk protein gene expression. Whatever the nature of the alterations in casein gene transcript stability induced by growth of COMMA-D cells on floating collagen gels, the preliminary results (not shown) of examining the rate of decay of steady-state β-casein mRNA in the presence of actinomycin D suggest that the stability of β-casein mRNA in the cytoplasm appears to be at best minimally affected by the substratum on which the cells are grown and cannot account for the much larger changes in mRNA accumulation. As such, these results suggest that alterations in the processing or stability of β-casein RNA in the nucleus are in part responsible for the effect of cell substratum on β-casein gene expression. It is of interest that other studies in our laboratory (47a) also suggest that prolactin affects β-casein RNA stability primarily in the nucleus and not by altering the half-life of cytoplasmic casein mRNA.

The effect of growing COMMA-D cells on different substrata is a general one in that expression of genes, in addition to the caseins, whose protein products are more prevalent in the mammary gland during midpregnancy or lactation (8, 14, 36, 37) is also increased in COMMA-D cells grown on collagen gels. However, of equal importance was that this effect was selective, as expression of actin and histone mRNAs decreased in cells grown on collagen gels, probably reflecting a greatly decreased rate of growth and increased degree of differentiation of these cells. Cytoplasmic β-actin and histone gene expression has been shown to be positively regulated by growth rate in other cell types (21, 25). Also, when 3T3 preadipocytes are induced to differentiate, expression of actin and tubulin decreases (50).

How might cell substratum interactions affect gene expression? Growth of various cell types on collagenous substrata, on other extracellular matrices (ECMs), or in combination with other cell types affects their state of differentiation (18, 31, 34, 35, 40, 44, 51). One important change lies in the ability of MEC grown on collagen gels to synthesize and compartmentalize a complex basement membrane, the composition of which resembles that of the mammary gland ECM in vivo (46). Primary MEC on plastic deposit much less basement material and instead degrade much of the ECM components they synthesize. Furthermore, growth of MEC cells on complex extracts of ECM (6, 35, 40; Rosen et al., in press) or specific components of the ECM, such as laminin or heparan sulfate proteoglycan, promote mammary cell differentiation. These results suggest that the interaction of MEC with exogenously or endogenously supplied components of the ECM stimulates milk protein gene expression.

Cells contain receptors on their surface for components of the ECM (29). These receptors can act as a linkage, along with other molecules (27), between components of the ECM and components of the cytoskeleton (5, 29). The observation that occupation of the ECM receptor integrin (42) promotes myoblast differentiation, as well as evidence that interaction of preadipocytes with fibronectin inhibits their differentiation (51) and that growth of hepatocytes on various components of the ECM can affect their state of differentiation (18), demonstrates the effect of specific components of the ECM on cell differentiation. Bissell et al. (5) hypothesized that the ECM might influence gene expression by affecting the organization of the cytoplasm, probably via alterations in the cytoskeleton. They further hypothesized that this either might involve modulations in the cytoplasmic stability of RNAs or, through effects of the cytoskeleton on some aspect of nuclear structure, might affect the processing or stability of nuclear RNAs. Although we are far from being able to clearly define the mechanisms by which ECM promotes casein RNA accumulation, our results suggest that it is in part due to regulation of casein RNA transcript stability within the nucleus. Our studies on MEC as well as results from the work of Fujita and co-workers (18) on hepatocytes indicate that cell-substratum interactions regulate tissue-specific gene expression at the posttranscriptional level.

In addition to the differential hormonal and developmental regulation of β-casein and WAP genes, expression of these genes is differentially regulated by cell-substratum interactions. In primary mouse MEC, WAP mRNA is initially present at high levels but rapidly disappears and remains undetectable irrespective of the substratum on which the cells are grown (32, 35). We were able to detect low levels of intact WAP mRNA in COMMA-D cells. With kinase-labeled RNA and runoff transcription assays, the WAP gene was expressed in a substratum-independent fashion at a level similar to that of the β-casein gene in cells grown on floating collagen. It appears that the WAP gene is transcribed constitutively but that WAP mRNA transcripts are unstable. A somewhat similar phenomenon in C2 hepatoma cells, in which PEPCK and phenylalanine hydroxylase gene transcription occurs at a high rate, but their intact mRNAs are undetectable (17).

Hormonal regulation of casein gene expression. In order to more clearly define the role of M, F, and I on casein gene expression, we used horse serum that was stripped of

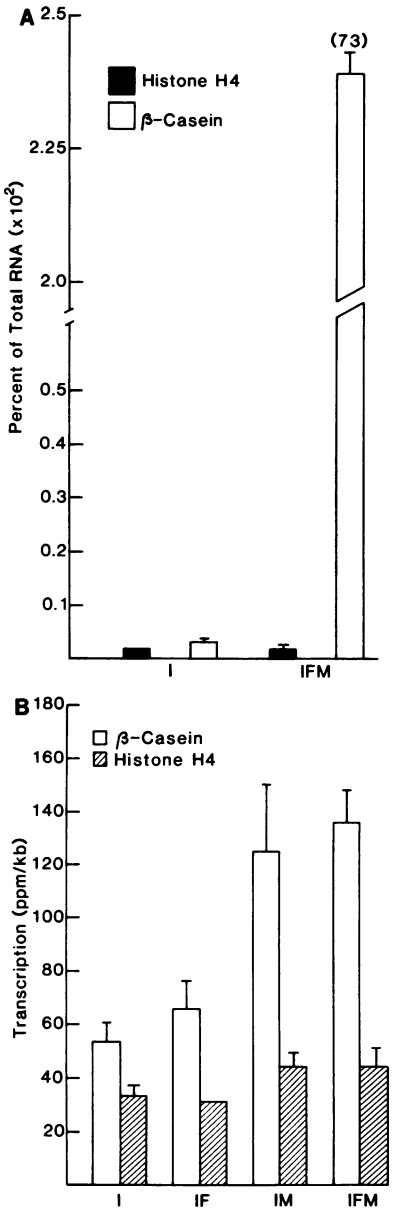

FIG. 3. (A) Hormonal induction of β-casein mRNA accumulation in COMMA-D cells. COMMA-D cells were grown in the presence of I plus medium containing charcoal-dextran-stripped horse serum instead of FCS serum on attached collagen gels for 48 h and then on floating collagen gels for a total of 72 h. During the final 48 h, the medium was changed to one containing stripped horse serum plus I alone or IFM. At the end of this period, total RNA was isolated, and measurement of casein and histone mRNA levels was performed as described in the legend to Fig. 1A. Values are expressed as mean ± SEM for triplicate hybridizations. (B) Effect of individual lactogenic hormones on β-casein and histone H4 gene transcription. COMMA-D cells were grown on floating collagen gels as described for panel A. After 48 h in the presence of I or IFM, nuclei were isolated and used for in vitro transcription assays. Values are expressed as mean ± SEM for triplicate hybridizations.

endogenous steroids. When COMMA-D cells were grown on floating collagen gels in the presence of I alone, β-casein mRNA was approximately as abundant as histone H4 mRNA (Fig. 3A). However, when all three hormones were present (IFM) for a 48-h period, a 73-fold increase in β-casein mRNA was noted, with no change in histone H4 mRNA concentration compared with the levels in cells grown with I alone. Compared with the control (I alone), cells grown in the presence of IF for 48 h exhibited no increase in β-casein mRNA levels, while growth in the presence of IM for 48 h increased the β-casein mRNA concentration two- to threefold (not shown).

The sensitivity of our transcription measurements allowed us to detect casein gene transcription under conditions in which, in other systems (10, 11), it had previously been found to be undetectable. β-Casein gene transcription was 55 ppm/kilobase (kb) in cells grown in the presence of I alone (Fig. 3B). No significant change in β-casein gene transcription was observed with the addition of F, but in the presence of IM the rate of transcription increased to 130 to 160 ppm/kb. Addition of all three hormones in combination (IMF) stimulated β-casein transcription to the same extent as IM alone. Together, these results bring forth several new points concerning the hormonal regulation of β-casein gene expression. First, in I alone, a significant level of β-casein gene transcription was observed. Second, F had no further effect on β-casein gene transcription (Fig. 3B) or mRNA accumulation (data not shown). Third, addition of M in the presence of I stimulated both β-casein gene transcription (Fig. 3B) and mRNA accumulation by two- to threefold (not shown).

We examined the kinetics of the effect of IFM on β-casein gene transcription and mRNA accumulation. β-Casein mRNA levels increased by 4 h after addition of FM to cells previously grown in I alone and continued to accumulate for at least 48 h (Fig. 4A). However, β-casein gene transcription was not affected until 48 h after addition of FM (Fig. 4B). At 36 and 72 h after addition of FM, β-casein gene transcription was stimulated to the same extent as the 48 h time point (not shown). Thus, hormone-dependent alterations in the post-transcriptional regulation of β-casein gene expression precede the increase in β-casein gene transcription.

I is required to obtain maximal levels of milk protein gene expression (10, 53). In the COMMA-D system, we were able to measure β-casein gene transcription in the presence of I alone and found that it was about one-third of the rate measured in the presence of IFM. Our results are the first demonstration of active transcription of the casein genes in the presence of I alone. Additional studies are required to determine whether I alone or other as yet unidentified factors present in the stripped horse serum are required to maintain the basal rate of β-casein gene transcription. Given the markedly decreased level of β-casein mRNA under these conditions, it appears that in the absence of M and F, β-casein gene transcription occurs but casein RNA transcripts are highly unstable.

Previous studies have revealed that glucocorticoids, like M, act to prolong the half-life of casein RNA transcripts (11, 28). Others (11) have concluded that F stimulates casein gene transcription. However, given the facts that in this study (11), both total and specific transcription was decreased in the absence of F, that transcription of control genes was not examined, and that casein transcription was detectable only in the presence of all three hormones, it is difficult to assess the specificity of these results. We found no effect of F on β-casein transcription, but there was an absolute requirement for this hormone in order to obtain maximal induction of casein mRNA. Some evidence suggests that glucocorticoids may not directly affect casein gene expression. First, our results indicate there was no effect of F on β-casein transcription. Second, protein synthesis is

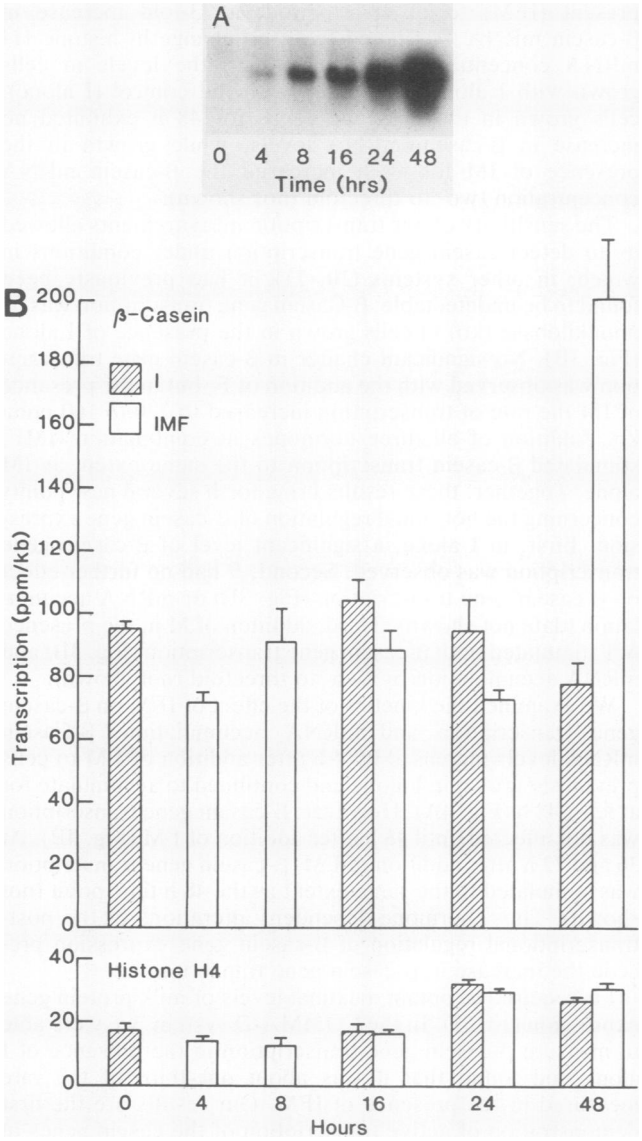

FIG. 4. (A) Time-dependent induction of β-casein mRNA accumulation by lactogenic hormones. Cells were grown as described in the text. At each time point, cells were harvested and used for total RNA isolation and RNA blotting. A 10-μg amount of RNA was applied per lane. A ^{32}P-labeled antisense RNA probe was used. Time points 0, 4, 8, 16, 24, and 48 h refer to time after addition of MF. (B) Time-dependent effect of lactogenic hormones on β-casein and histone H4 gene transcription. Cells were grown and harvested as described for panel A. In vitro transcription assays were performed with nuclei isolated from cells grown in I alone (0 h) or in IFM for an additional 4, 8, 16, 24, or 48 h. Values are expressed as mean ± SEM for triplicate hybridizations.

required for F to stimulate β-casein mRNA accumulation (Rosen et al., in press), and there is a 3-h lag in the effect of F on casein mRNA accumulation in mammary tissue explants (19). Finally, linoleic acid can replace glucocorticoids in the stimulation of casein protein accumulation in MEC grown in serum-free collagen gel cultures (33). From these findings, it appears that glucocorticoids influence casein gene expression solely at the posttranscriptional level.

In mammary tissue explants, M in the presence of F and I stimulates β-casein gene transcription two- to threefold as measured by incorporation of [^{3}H]uridine (24). By examining β-casein transcription in COMMA-D cells in the presence of I alone, IF, IM, and IFM, it is now possible to state that the increase in β-casein transcription caused by IFM is induced by M. Addition of M to cells grown with I stimulated β-casein transcription and mRNA accumulation to a similar extent. Thus, the effect of M in the presence of I alone on β-casein mRNA accumulation appears to be exerted primarily at the transcriptional level. Our results, as well as those of others (10, 11, 28), suggest that regulation of β-casein RNA stability requires the simultaneous presence of both M and F. We demonstrated that this effect of M and F on β-casein RNA stability occurred before the effect of M on casein gene transcription, showing again the predominant role of RNA stability, not gene transcription, in the hormone-dependent accumulation of β-casein RNA.

The increased sensitivity of our transcription measurements allowed us to conclude that both the hormonal and cell substratum induction of β-casein mRNA involved similar types of regulation. Both of these influences on β-casein gene expression involved a two- to threefold stimulation of transcription with a much larger increase in RNA stability. These changes in β-casein RNA stability apparently do not occur in the cytoplasm. Together, these results lead us to speculate that these effectors, both of which act through cell surface receptors, might have similar mechanisms of action. These could include alterations in β-casein gene topology involving its association with the nuclear matrix or the accessibility of β-casein genes or their RNA transcripts to various *trans*-acting factors. In order to address these issues, we are examining the effects of hormones and cell substratum on the metabolism of β-casein RNA within the nucleus.

ACKNOWLEDGMENTS

These studies were supported by Public Health Service grant CA-16303 from the National Institutes of Health to J.M.R. and by a postdoctoral fellowship from the American Cancer Society (PF-2782) to R.S.E.

LITERATURE CITED

1. **Bagchi, S., L. S. Wise, M. L. Brown, D. Bregman, H. S. Sul, and C. S. Rubin.** 1987. Structure and expression of murine malic enzyme mRNA. J. Biol. Chem. **262:**1558–1565.
2. **Beale, E. G., N. Chrapkiewicz, H. A. Scoble, R. J. Metz, D. P. Quick, R. L. Noble, J. E. Donelson, K. Biemann, and D. K. Granner.** 1985. Rat hepatic cytosolic phosphoenolpyruvate carboxykinase (GTP). J. Biol. Chem. **260:**10748–10760.
3. **Bergsma, D. J., K. S. Chang, and R. J. Schwartz.** 1985. Novel chicken actin gene: third cytoplasmic isoform. Mol. Cell. Biol. **5:**1151–1162.
4. **Bisbee, C. A., and J. M. Rosen.** 1987. DNA sequence elements regulating casein gene expression. UCLA Symp. Mol. Cell. Biol. New Ser. **52:**313–323.
5. **Bissell, M. J., H. G. Hall, and G. Parry.** 1982. How does the extracellular matrix direct gene expression? J. Theor. Biol. **99:** 31–68.
6. **Blum, J. L., M. E. Zeigler, and M. S. Wicha.** 1987. Regulation of rat mammary gene expression by extracellular matrix components. Exp. Cell Res. **173:**322–340.
7. **Campbell, S. M., J. M. Rosen, L. G. Hennighausen, U. Strech-Jurk and A. E. Sippel.** 1984. Comparison of the whey acidic protein genes of the rat and mouse. Nucleic Acids. Res. **12:** 8685–8697.
8. **Chen, L.-H., and M. J. Bissell.** 1987. Transferrin mRNA level in the mouse mammary gland is regulated by pregnancy and extracellular matrix. J. Biol. Chem. **262:**17247–17250.
9. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active RNA from sources enriched in ribonucleases. Biochemistry **18:**5294–5299.

10. **Chomczynski, P., P. Qasba, and Y. J. Topper.** 1984. Essential role of insulin in transcription of the rat 25,000 molecular weight casein gene. Science **222**:1326–1328.
11. **Chomczynski, P., P. Qasba, and Y. J. Topper.** 1986. Transcriptional and post-transcriptional roles of glucocorticoid in the expression of the rat 25,000 molecular weight casein gene. Biochem. Biophys. Res. Commun. **134**:812–818.
12. **Danielson, K. G., C. J. Oborn, E. M. Durban, J. S. Butel, and D. Medina.** 1984. Epithelial mouse mammary cell line exhibiting normal morphogenesis in vivo and functional differentiation in vitro. Proc. Natl. Acad. Sci. USA **81**:3756–3760.
13. **Devinoy, E., L.-M. Houdebine, and C. Delois.** 1978. Role of prolactin and glucocorticoid in the expression of casein genes in rabbit mammary gland organ culture. Biochim. Biophys. Acta **517**:360–366.
14. **Emerman, J. T., J. C. Bartley, and M. J. Bissell.** 1981. Glucose metabolite patterns as markers of functional differentiation in freshly isolated and cultured mouse mammary epithelial cells. Exp. Cell Res. **134**:241–250.
15. **Emerman, J. T., and D. R. Pitelka.** 1977. Maintenance and induction of morphological differentiation in dissociated mammary epithelium on floating collagen membranes. In Vitro **13**: 316–328.
16. **Emerman, J. T., S. J. Burwen, and D. R. Pitelka.** 1979. Substrate properties influencing ultrastructural differentiation of mammary epithelial cells in culture. Tissue Cell **11**:109–119.
17. **Friedman, J. M., L. E. Babiss, M. Weiss, and J. E. Darnell, Jr.** 1987. Hepatoma variants (C2) are defective for transcriptional and posttranscriptional actions from both endogenous and viral genomes. EMBO J. **6**:1727–1731.
18. **Fujita, M., D. C. Spray, H. Choi, J. Saez, D. M. Jefferson, E. Hertzberg, L. C. Rosenberg, and L. M. Reid.** 1986. Extracellular matrix regulation of cell-cell communication and tissue specific gene expression in primary liver cultures. p. 333–360. *In* G. Serrero and J. Hayashi (ed.), Cellular endocrinology: hormonal control of embryonic and cellular differentiation. Alan R. Liss, New York.
19. **Ganguly, R., N. Ganguly, N. M. Mehta, and M. R. Banerjee.** 1980. Absolute requirement of glucocorticoid for expression of the casein gene in the presence of prolactin. Proc. Natl. Acad. Sci. USA **77**:6003–6006.
20. **Greenberg, M. E., and E. B. Ziff.** 1984. Stimulation of 3T3 cells induces transcription of the c-*fos* proto-oncogene. Nature (London) **311**:433–438.
21. **Greenberg, M. E., A. L. Hermanowski, and E. B. Ziff.** 1986. Effect of protein synthesis inhibitors on growth factor activation of c-*fos*, c-*myc*, and actin gene transcription. Mol. Cell. Biol. **6**: 1050–1057.
22. **Grosschedl, R., D. Weaver, D. Baltimore, and F. Costantini.** 1984. Introduction of a μ immunoglobulin gene into the mouse germ line: specific expression in lymphoid cells and synthesis of functional antibody. Cell **38**:647–658.
23. **Gupta, P., J. M. Rosen, P. D'Eustachio, and F. H. Ruddle.** 1982. Localization of the casein gene family to a single mouse chromosome. J. Cell Biol. **93**:199–204.
24. **Guyette, W. A., R. J. Matusik, and J. M. Rosen.** 1979. Prolactin mediated transcriptional and posttranscriptional regulation of casein gene expression. Cell **17**:1013–1023.
25. **Heintz, N., H. L. Sive, and R. G. Roeder.** 1983. Regulation of human histone gene expression: kinetics of accumulation and changes in rate of synthesis and in half-lifes of individual histone mRNAs during the HeLa cell cycle. Mol. Cell. Biol. **3**:539–550.
26. **Hobbs, A. A., A. Richards, D. J. Kessler, and J. M. Rosen.** 1982. Complex hormonal regulation of rat casein gene expression. J. Biol. Chem. **257**:3598–3605.
27. **Horwitz, A., K. Duggan, C. Buck, M. C. Beckerle, and K. Burridge.** 1986. Interaction of plasma membrane fibronectin receptor with talin—a transmembrane linkage. Nature (London) **320**:531–533.
28. **Houdebine, L.-M., E. Devinoy, and C. Delouis.** 1978. Stabilization of casein mRNA by prolactin and glucocorticoids. Biochimie **60**:57–63.
29. **Hynes, R. O.** 1987. Integrins: a family of cell surface receptors. Cell **48**:549–554.
30. **Israel, D. I., and J. P. Whitlock, Jr.** 1984. Regulation of cytochrome P_1-450 gene transcription by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in wild type and variant mouse hepatoma cells. J. Biol. Chem. **259**:5400–5402.
31. **Kleinman, H. K., L. Luckenbill-Edds, F. W. Cannon, and G. C. Sephel.** 1987. Use of extracellular matrix components for cell culture. Anal. Biochem. **166**:1–13.
32. **Lee, E. Y.-H., G. Parry, and M. J. Bissell.** 1984. Modulation of secreted protein of mouse mammary epithelial cells by collagenous substrata. J. Cell Biol. **98**:146–155.
33. **Levay-Young, B. K., G. K. Bandyopadhyay, and S. Nandi.** 1987. Linoleic acid, but not cortisol, stimulates accumulation of casein by mouse mammary epithelial cells in serum-free collagen gel culture. Proc. Natl. Acad. Sci. USA **84**:8448–8452.
34. **Levine, J. F., and F. E. Stockdale.** 1985. Cell-cell interactions promote mammary epithelial cell differentiation. J. Cell. Biol. **100**:1415–1422.
35. **Li, M. L., J. Aggeler, D. A. Farson, C. Hatier, J. Hassell, and M. J. Bissell.** 1987. Influence of a reconstituted basement membrane and its components in casein gene expression and secretion in mouse mammary epithelial cells. Proc. Natl. Acad. Sci. USA **84**:136–140.
36. **Lobato, M. F., M. Careche, M. Ros, F. J. Moreno, and J. P. Garcia-Ruiz.** 1985. Effect of prolactin and glucocorticoids on P-enolpyruvate carboxykinase activity in liver and mammary gland from diabetic and lactating rats. Mol. Cell. Biochem. **67**: 19–23.
37. **Lobato, M. F., M. Ros, M. J. Moreno, and J. P. Garcia-Ruiz.** 1986. Nutritional and hormonal regulation of malic enzyme synthesis in rat mammary gland. Biochem. J. **236**:441–445.
38. **McKnight, G. S., and R. D. Palmiter.** 1979. Transcriptional regulation of the ovalalbumin and conalbumin genes by steroid hormones in chick oviduct. J. Biol. Chem. **254**:9050–9058.
39. **McMaster, G. K., and G. G. Carmichael.** 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels using glyoxal and acridine orange. Proc. Natl. Acad. Sci. USA **74**:4835–4839.
40. **Medina, D., M.-L. Li, C. J. Oborn, and M. J. Bissell.** 1987. Casein gene expression in mouse mammary epithelial cell lines: dependence upon extracellular matrix and cell type. Exp. Cell Res. **172**:192–203.
41. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
42. **Menko, A. S., and D. Boettiger.** 1987. Occupation of the extracellular matrix receptor, integrin, is a control point for myogenic differentiation. Cell **51**:51–57.
43. **Murdoch, G. H., E. Potter, A. K. Nicolaisen, R. M. Evans, and M. G. Rosenfeld.** 1982. Epidermal growth factor rapidly stimulates prolactin gene transcription. Nature (London) **300**:192–194.
44. **Muschel, R., G. Khoury, and L. M. Reid.** 1986. Regulation of insulin mRNA abundance and adenylation: dependence on hormones and matrix substrata. Mol. Cell. Biol. **6**:337–341.
45. **Parry, G., B. Cullen, C. S. Kaetzel, R. Kramer, and L. Moss.** 1987. Regulation of differentiation and polarized secretion in mammary epithelial cells maintained in culture: extracellular matrix and membrane polarity influences. J. Cell Biol. **105**: 2043–2051.
46. **Parry, G., E. Y.-H. Lee, D. Farson, M. Koval, and M. J. Bissell.** 1985. Collagenous substrata regulate the nature and distribution of glycosaminoglycans produced by differentiated cultures of mouse mammary epithelial cells. Exp. Cell Res. **156**:487–499.
47. **Rodgers, J. R., M. L. Johnson, and J. M. Rosen.** 1985. Measurement of mRNA concentration and mRNA half-life as a function of hormonal treatment. Methods Enzymol. **109**:572–592.
47a. **Rosen, J. M., J. R. Rogers, C. H. Couch, C. A. Bisbee, Y. David-**

Inouye, S. M. Campbell, and L.-Y. Yu-Lee. 1986. Multihormonal regulation of milk protein gene expression. Ann. N.Y. Acad. Sci. **478**:63–76.

48. **Schwartz, L. B., V.E.F. Sklar, J. A. Jaehning, R. Weinmann, and R. Roeder.** 1974. Isolation and partial characterization of the multiple forms of deoxyribonucleic acid-dependent ribonucleic acid polymerase in the mouse myeloma, MOPC 315. J. Biol. Chem. **249**:5889–5897.
49. **Shannon, J. M., and D. R. Pitelka.** 1981. The influence of cell shape on the induction of functional differentiation on mouse mammary cell in vitro. In Vitro **17**:1016–1028.
50. **Spiegelman, B., and S. R. Farmer.** 1982. Decreases in tubulin and actin gene expression prior to morphological differentiation of 3T3-adipocytes. Cell **29**:53–60.
51. **Spiegelman, B. M., and C. A. Ginty.** 1983. Fibronectin modulation of cell shape and lipogenic gene expression in 3T3-adipocytes. Cell **35**:657–666.
52. **Suard, Y. M. L., M.-T. Haeuptle, E. Farinon, and J.-M. Kraehenbuhl.** 1983. Cell proliferation and milk protein gene expression in rabbit mammary cell cultures. J. Cell Biol. **96**: 1435–1442.
53. **Topper, Y. J., and C. S. Freeman.** 1980. Multiple hormone interactions in the developmental biology of the mammary gland. Physiol. Rev. **60**:1049–1106.
54. **Ucker, D. S., and K. R. Yamamoto.** 1984. Early events in the stimulation of mammary tumor virus RNA synthesis by glucocorticoids: novel assays of transcription rates. J. Biol. Chem. **259**:7416–7420.