Tissue-specific, high level expression of the rat whey acidic protein gene in transgenic mice

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ABSTRACT

The importance of intragenic and 3′ flanking sequences in the control of the temporal, hormonal and tissue-specific expression of milk whey acidic protein (WAP) has been demonstrated in transgenic mice. Mouse lines carrying a 4.3 kb genomic clone containing the entire rat WAP gene minus 200 bp of the first intron with 0.949 kb of 5′ and 1.4 kb of 3′ flanking DNA were generated. In eight of nine independent lines of mice analyzed, WAP transgene expression was detected at levels ranging from 1% to 95% (average, 27%) of the endogenous gene. The transgene was expressed preferentially in the mammary gland. Although developmentally regulated during pregnancy and lactation, the temporal pattern of WAP transgene expression differed from the endogenous gene. A precocious increase in expression of the transgene was detected at 7 days of pregnancy, several days earlier in pregnancy than the major increase observed in endogenous mouse WAP mRNA. The rat WAP transgene was translated and secreted into the milk of transgenic mice at levels comparable to the endogenous mouse WAP. This is the first report of a gene that is negatively regulated in dissociated cell cultures as well as in transfected cells, yet is expressed efficiently in the correct multicellular environment of the transgenic mouse.

INTRODUCTION

Mammary gland development during pregnancy and lactation involves cellular differentiation characterized by the expression of a number of tissue-specific genes, including those encoding various milk proteins (1). Milk protein gene expression is not only hormonally regulated, but is also dependent upon complex cell-cell, and cell-extracellular matrix interactions (2). The regulation of several mammary specific genes, notably β-casein, has been studied utilizing cell lines and primary mammary epithelial cell cultures (3−5). However, the major whey protein in rodent milk, whey acidic protein (WAP), usually is not expressed in dissociated cell cultures (6) or mammary cell lines (7), even though in the 8-day lactating mammary gland, WAP mRNA makes up 10−15% of the poly A(+) RNA (9). It has been reported recently that high levels of WAP expression in cell culture can be obtained in primary mouse mammary epithelial cells cultured on a basement membrane type matrix when 3-dimensional alveoli-like spherical structures are formed (10).

The developmental regulation of WAP gene expression in the mammary gland parallels that of the caseins, but increases somewhat more slowly during pregnancy. WAP gene expression is hormonally-regulated in explant cultures by glucocorticoids and prolactin, but is differentially regulated by these hormones as compared to casein gene expression (9). Perhaps the major difference between WAP and casein gene expression lies in their sensitivity to extracellular signals, since efficient WAP gene expression is only observed under conditions which maintain an organized cellular structure similar to that observed in the intact mammary gland (9,10).

The entire rat WAP gene and flanking DNA sequences have been isolated and characterized previously in our laboratory (11). To define sequences important for the hormonal and tissue-specific regulation of the WAP gene, a number of different permanently transfected mammary and non-mammary cell lines were generated carrying the entire gene with 0.949 kb of 5′ and 1.4 kb of 3′ flanking DNA and a small 200 bp deletion in the first intron. However, the transfected gene was not expressed in these experiments despite the presence of a strong viral enhancer in these constructs, indicating that either the gene was under negative regulation in the cell lines studied, or important cis-acting DNA sequences were missing from this construct (12).

In order to overcome the limitations imposed by the lack of permanent cell lines expressing the WAP gene, transgenic mice have been utilized to maintain the correct hormonal and structural environment necessary for the proper spatial and temporal expression of this gene (13,14,15). A 2.5 kb fragment containing the promotor and upstream sequences of the mouse WAP gene has demonstrated in a variety of constructs to target the expression of the oncogenes H-*ras* (13) and c-*myc* (15) as well as tissue plasminogen activator (tPA,16) to the mammary glands of transgenic mice. However, both the level and frequency of expression of these constructs have been quite low. These experiments have demonstrated that 5′ flanking sequences in the WAP gene contain the information necessary to specify both

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tissue-specific and developmental control of WAP gene expression, but that additional sequences required for the high levels of expression seen *in vivo* and for hormonal regulation must lie elsewhere (17). This conclusion has been reinforced by nuclear run-on transcription experiments, which suggest that a major level of control of WAP gene regulation is post-transcriptional (7,10, R. McKnight and L. Hennighausen, personal communication).

To determine the importance of intragenic as well as 3′ flanking DNA sequences in the regulation of WAP gene expression, transgenic mice containing the rat WAP gene have been generated. In contrast to cell transfection experiments, several independent lines of mice expressed the transgene at levels comparable to those observed for the endogenous mouse gene. The entire rat WAP transgene was developmentally regulated, translated and secreted into the milk of transgenic mice.

MATERIALS AND METHODS

Enzymes and Plasmids

All restriction enzymes, Klenow enzyme and T4 ligase were purchased from either Bethesda Research Laboratories (Bethesda, MD) or Boehringher Mannheim Biochemicals (Indianapolis, IN). Sp6 and T7 polymerase, Sp64 and pGem® 3Z plasmids and DNase I were purchased from Promega (Madison, WI). The plasmid template for the synthesis of the mouse WAP antisense RNA to the first intron was a gift from L. Hennighausen (16). RNase A was purchased from Sigma (St. Louis, MO.) and RNase T1 from Boehringer Mannheim Biochemicals. Enzymes were used according to suppliers' specifications.

Generation and Screening of Transgenic Mice

Rat WAP transgenic mice were generated by microinjection of a *Sal* I-*Acc* I linear sequence restriction fragment of the rat WAP gene containing 0.949 kb of 5′ and 1.4 kb of 3′ flanking sequences (Fig. 1). This construct contains a 200 bp deletion in the first intron denoted (−0.949/0.2)(0.4/3.5) in Campbell, 1986, Ph.D. Thesis, Baylor College of Medicine. The microinjection and transfer of mouse embryos were carried out as described previously as was the isolation of genomic DNA (18).

Genomic DNA was screened by Southern hybridization analysis as follows: approximately 15 μg of tail DNA was digested with *Bam*HI in the presence of 5 mM spermidine overnight (19), fractionated in a 1% agarose gel, alkaline transferred to Zeta-probe® (BioRad, Richmond, CA) nylon filters according to supplier's protocol, and hybridized to ^{32}P-labeled rat cDNA generated using random hexanucleotide primers (20,21). This probe hybridizes to a 3.1 kb rat genomic DNA fragment (Fig. 1, lanes A and B) and a 2.8 kb fragment from the injected transgene (Fig. 1, lanes C-M). The filter was washed according to the Zeta-probe® protocol and autoradiography was performed with Kodak XAR film and a DuPont intensifying screen at −80°. The copy number of the transgene was determined by comparing the intensity of the 3.1 kb *Bam*HI fragment from variable inputs of rat DNA measured with a fluorometric assay and quantitated by scanning densitometry to the 2.8 kb *Bam*HI fragment in a known amount of transgenic mouse DNA.

RNA Isolation

Total RNA was isolated by homogenization of tissues in a guanidium isothiocyanate solution followed by CsCl gradient fractionation using the method of Chirgwin *et al.* (22).

Preparation of Antisense RNA Probes

A 432 bp fragment of the rat cDNA starting 26 bp into the second exon was subcloned into the *Pst*I site of the polylinker of the pGem® 3Z vector. The plasmid was linearized at the *Nde*I site and antisense RNA labeled with [^{32}P] GTP to a specific activity of approximately 2×10^7 cpm/μg was produced with Sp6 polymerase. The mouse WAP cDNA 549 bp fragment starting 16 bp into the first exon cloned into the Sp64 vector was linearized at the *Pvu*II site and antisense RNA was synthesized as described above. The antisense probe from the mouse WAP gene 5′ end including the first exon was synthesized from a Bluescribe® vector as described by Pittius *et al.* (16). After synthesis the probes were purified on an Elutip-R® (Schleicher & Schuell, Keene, NH) column according to manufacturer's directions.

RNase Protection Assays

Mammary gland biopsies were performed on the female founder mice and the F_1 females from the male founder mice at 10 days of lactation. The fourth abdominal mammary gland was surgically removed under anesthesia and total RNA isolated as described. Total RNA isolated from mammary glands and other tissues was incubated with 200,000 cpm of ^{32}P-labeled antisense RNA (cRNA) under conditions of probe excess and using several RNA inputs essentially as described (16) except RNase H digestion was not necessary since the probes were purified on Elutip-R® columns. Electrophoresis was performed on 3% polyacrylamide/8 M urea gels for the larger cRNA probes and 6% for the smaller first exon probe. The individual lanes containing the protected fragments were excised and radioactivity determined by liquid scintillation counting to quantitate the levels of expression. The data obtained for the protected fragment derived from the rat WAP cRNA probe were corrected for the fewer number of labeled guanosines present as compared to the protected fragment derived from the mouse cRNA probe (113 for the rat and 163 for the mouse).

Collection and Fractionation of Milk

Milk was collected from transgenic mice at 10 days of lactation and processed as described (23) to obtain skim milk. The skim milk was diluted in Tris-saline solution (0.125 M NaCl, 25 mM Tris-HCl, pH 7.4, 5 mM KCl) containing phenylmethylsulfonyl-fluoride (2 mM) and stored at −70°. Two-dimensional gel electrophoresis was performed according to Dunbar, 1987 (24) as follows: the skim milk proteins were dissolved in urea with 2% β-mercaptoethanol and ultracentrifuged at 36,000 rpm in a Ti 42.2 rotor for 1 h. The first dimension isoelectric focusing gels were electrophoresed at 700V for 17 h. The separation in the second dimension was through a 10−20% gradient SDS-polyacrylamide gel with stepwise increasing voltage from 200−450V over a period of 4 h.

RESULTS

Production of transgenic mice containing the rat WAP gene

Transgenic mice containing the entire rat WAP gene were produced by microinjection of a vector-free, 4.3 kb genomic DNA fragment of the WAP gene (Fig. 1). The entire WAP gene was injected since there is evidence that WAP gene expression is regulated at the post-transcriptional level in mammary cells (7,10) and numerous regulatory elements have been found located within introns (25−28) as well as 3′ flanking regions of genes (29,30). Furthermore, introns may affect the level of expression of transgenes in mice (31).

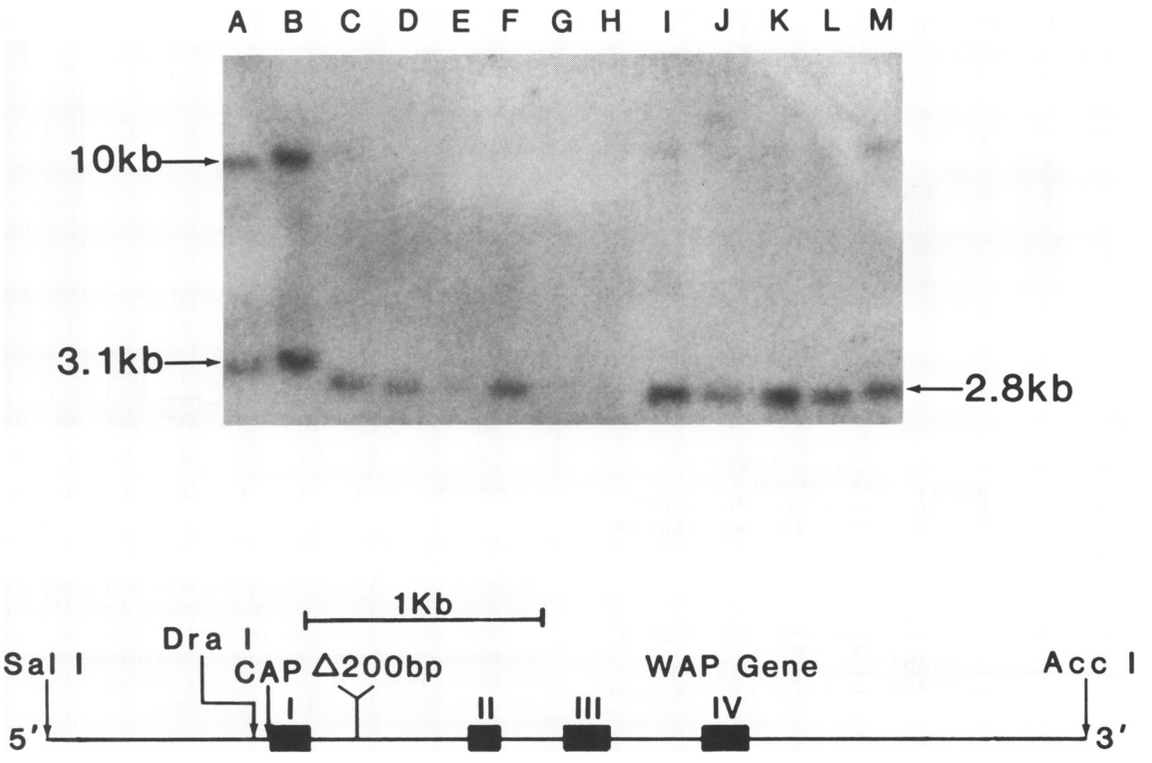

Figure 1. Southern hybridization analysis of restriction enzyme digests from mouse genomic DNA. DNA was digested with *Bam*HI and probed with ^{32}P-labeled rat WAP cDNA. Lanes A and B represent 10 and 20 μg, respectively, of rat genomic DNA. Lanes C-M contain 5 μg each of DNA for 11 lines of transgenic mice. The bands at 10 kb represent hybridization with the junction fragment from the *Bam*HI digest and the endogenous mouse WAP gene. Note the difference in size between the endogenous rat fragment (3.1 kb) and the transgene fragment (2.8) showing the deletion in the injected gene construct. Below: The structure of the rat WAP transgene indicating the 200 bp deletion within the first intron. The 4.3 kb transgene contains the structural gene as well as 0.949 kb of 5′ and 1.4 kb of 3′ flanking sequences.

Analysis of genomic tail DNA following restriction enzyme digestion with *Bam*HI and hybridization with random hexanucleotide primed rat WAP cDNA probes was employed to distinguish the rat transgene from the endogenous mouse WAP gene (Fig. 1). A specific 2.8 kb band was detected in the positive transgenics (Fig. 1, lanes C−M), which is the size expected resulting from the 200 bp deletion in the first intron of the transgene, as compared to the 3.1 kb band observed in rat genomic DNA (Fig. 1, lanes A,B). The 10 kb bands detected with the rat WAP cDNA probe represent the junction fragment from the *Bam*HI restriction enzyme digestion as seen in rat genomic DNA (Fig. 1, lanes A,B) and the endogenous mouse WAP gene (data not shown). Twelve positive independent lines carrying the transgene were obtained from 63 mice screened with copy numbers ranging from 2 to 8 copies of the transgene, determined as described in *Materials and Methods*. All of these founder mice transmitted the transgene to their offspring in a Mendelian fashion. Nine lines were employed for further expression studies.

Expression of the Rat WAP Transgene

In order to quantitate the expression of the transgene as well as the endogenous mouse WAP gene, RNA isolated from mammary glands of mice at 10 days of lactation was assayed using the RNase protection protocol described in *Materials and Methods*. For detection of the rat transgene, the 762 nucleotide cRNA probe (Fig. 4B, lane 1) protected approximately 450 nucleotides (NT) of the rat WAP mRNA (Fig. 2). This protected fragment was compared to the 550 NT protected fragment of the endogenous mouse WAP mRNA. Yeast tRNA (Fig. 2, lane B), RNA isolated from non-transgenic mice at 10 days of lactation hybridized with the rat WAP cRNA probe (Fig. 6, lane C), and RNA isolated from rat mammary glands at mid-lactation hybridized with the mouse WAP cRNA probe (Fig. 2, lane C) were included as controls for nonspecific background radioactivity. There was very little crossreactivity observed as can be seen in Figure 2, lane C and Figure 6, lane C.

The relative levels of WAP transgene expression for several female offspring derived from male founder mice were averaged and the results are presented together with those obtained for biopsies from individual female founders (Fig. 3). In previous studies performed in transgenic mice using either mouse WAP promotor constructs or the entire rat β-casein gene, the expression of the transgenes was usually less than 1% of the level of the endogenous gene (16,18). However, reasonably high levels of WAP transgene expression were detected at 10 days of lactation in eight independent lines of mice at levels ranging from a minimum of 1% to 95% of the endogenous mouse gene, with an average of 27%. In only one line assayed was detectable expression not observed. The expression of the transgenes did not appear to be related to their copy number. For example, the highest expressing line, #5440, expressing 95% of the endogenous mouse WAP, contained only 3 copies of the transgene, as compared to line #5472 with 7 copies, whose expression was only 23% of the endogenous mouse WAP. These results indicate that the entire rat WAP gene when injected into mice is capable of being expressed at levels comparable to the

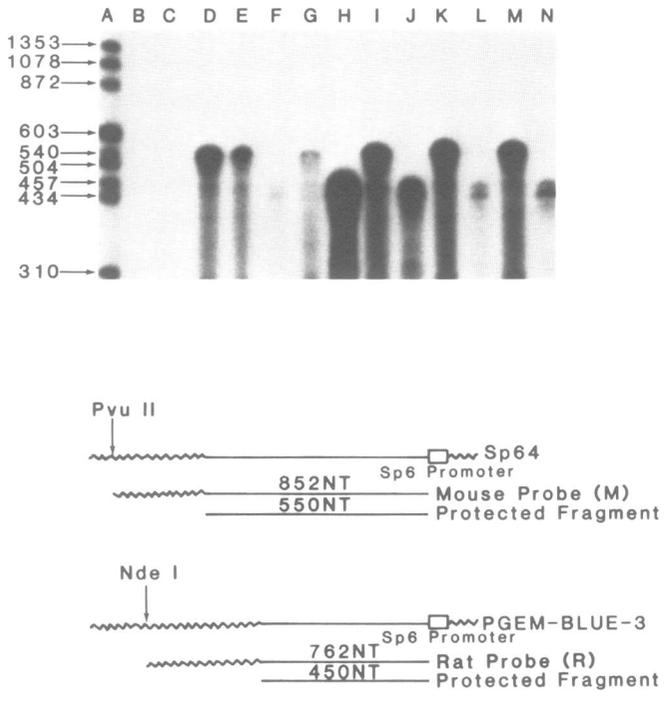

Figure 2. Analysis of rat transgenic and mouse endogenous WAP gene expression using an RNase protection assay. Lane A, ^{32}P-kinase labeled ΦX174-*Hae* III-digested fragments as markers (sizes in bp); B, yeast tRNA; C, RNA isolated from rat mammary gland at 13 days of lactation; D, RNA isolated from the mammary gland of a non-transgenic mouse at day 10 of lactation; E,G,I,K, and M, 1 μg total RNA from 3 different lines of transgenic mice (K and M are F_1 offspring of E) using a mouse cRNA probe; F and H (5 μg); J,L and N (1 μg) are from the same RNA samples as above only hybridized with a rat cRNA probe. Below: Diagrams of probes used in RNase protection assays and expected protected fragments.

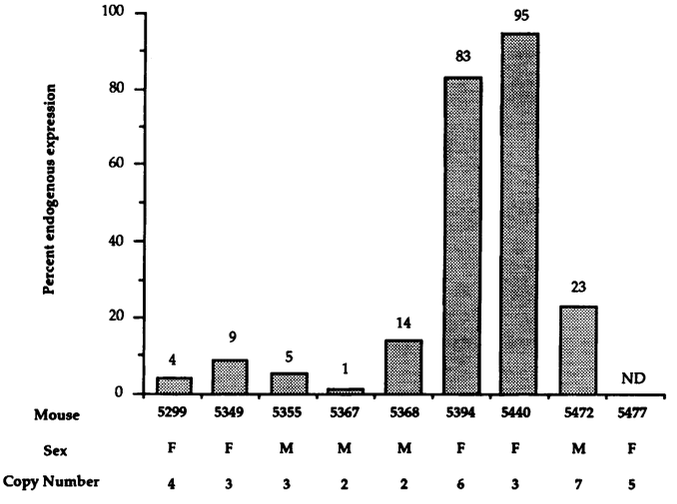

Figure 3. Summary of the relative level of rat WAP transgene expression. RNA was analyzed using the RNase protection assay as described in Fig. 2 and *Materials and Methods*. The amount of expression was measured by excising the protected fragments for both genes and counting in a scintillation counter. The percent transgene expression of the endogenous gene was calculated as described in *Materials and Methods*. ND; none detected.

endogenous mouse WAP gene, but that expression is neither position independent nor copy number dependent.

Tissue Specificity of the Rat WAP Transgene

In order to determine if the 4.3 kb rat WAP gene construct displayed a similar pattern of tissue specificity as the endogenous gene, total RNA was isolated from various tissues at day 10 of lactation from two independent lines of transgenic mice that displayed an order of magnitude different levels of expression (Lines #5394 and #5349) and analyzed using the RNase protection assay. A different mouse cRNA probe was employed for this analysis which increased the sensitivity of the assay and detected levels of expression in tissues that were several orders of magnitude less than those observed in the mammary gland. The cRNA probe used was a 322 NT fragment spanning from nucleotide −88 to +234 within the first intron of the mouse WAP gene (Fig. 4A, lane 1). This probe protects a 112 NT fragment that corresponds to the size of the first exon of the mouse WAP gene (16). This shorter mouse cRNA probe also crossreacted with the rat WAP transcripts as shown in Figure 4A, lane 3, but the protected fragment was much smaller (≈61 NT), presumably corresponding to transcripts of a homologous region between the first exons of the rat and mouse genes. Thus, this single mouse cRNA probe was able to distinguish between the expression of endogenous WAP and the transgene. To increase the sensitivity of the assay, the shorter cRNA probe was synthesized with a higher specific activity (1×10^9 cpm/μg). This probe generated a significantly lower nonspecific background than that observed using the larger 550 bp mouse cRNA probe. The specific activity of the rat cRNA probe also was increased in these assays to provide comparable sensitivity for the detection of transgene expression.

Under these conditions, the expression of the endogenous mouse WAP mRNA was detected in the tongue and the kidney of Line #5394 (Fig. 4A, lanes 11 and 12 respectively). Both the tongue and kidney, as well as the mammary gland show a band corresponding to the region of cross-reactivity with rat WAP mRNA transcripts observed in RNA isolated from the mammary gland of rats at day 10 of lactation (Fig. 4A, lanes 11,12,15, and 3 respectively) indicating expression of the rat transgene.

To determine if the cross-reactivity seen with the mouse-specific cRNA probe was indicative of authentic transgene expression and to quantitate the relative level of expression, these same RNAs were analyzed using the rat WAP cRNA probe and shown in Fig. 4B. Once again expression of the rat WAP transgene was detected in the kidney at a level estimated to be 5,000-fold less than that observed in the mammary gland. Similar expression in the kidney was observed in another mouse line, #5349 (data not shown). Expression of the transgene in the tongue could not be determined for this mouse line #5394 because of a lack of RNA, but mouse line, #5349, did not express either the endogenous or transgene in the tongue (data not shown).

The similar tissue expression pattern of the endogenous mouse and 4.3 kb rat WAP gene indicates that the rat transgene is regulated in a tissue-specific manner during lactation. Since the kinetics of induction of milk protein gene expression during pregnancy are not identical, it was of interest to examine whether the developmental expression pattern of the rat WAP transgene was similar to the endogenous WAP gene. There has been a previous report in transgenic mice of a precocious increase during pregnancy in the expression of a WAP promoter-driven reporter gene as compared to the endogenous WAP gene (17).

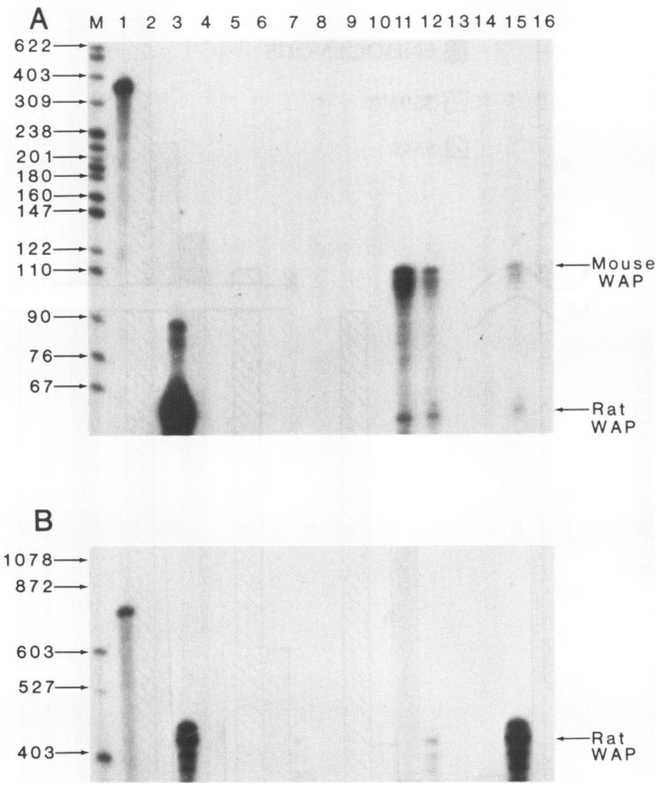

Figure 4. Tissue-specific expression of the mouse (A) and rat (B) WAP genes in transgenic mouse line #5394. RNA was hybridized with ^{32}P-labeled RNA complementary to the first exon of the mouse WAP gene (A) or to the rat WAP cRNA probe (B) and following RNase digestion the reaction products were separated on 6% (A) and 3% (B) polyacrylamide/8M urea sequencing gels as described in *Materials and Methods*. The arrows indicate the 112 bp fragment encoding the first exon of the endogenous mouse WAP gene and the cross-reacting 61 NT fragment of the rat WAP transgene (A) as well as the 450 bp fragment of the rat WAP cDNA encoding the last three exons of the rat WAP gene (B). The size markers (lanes M; sizes in bp) are a mixture of ^{32}P-labeled ΦX174 digested with *Hae* III and pBR322 digested with *Hpa* II. Panel A, lane 1, 322 NT cRNA probe for the endogenous mouse WAP; panel B, lane 1, 762 NT cRNA probe specific for the transgene. Panels A and B, lane 2, 10 μg yeast tRNA; Panel A and B, lane 15, 10^{-3} and 10^{-1} μg, respectively of RNA isolated from the mammary glands of transgenic mice at 10 days of lactation. Panel A and B, lane 3, 10 and 0.1 μg of RNA, respectively, isolated from the mammary gland of rats at day 13 of lactation. All other lanes, except 16, contain 10 μg of total RNA from various tissues of a female transgenic mouse at 10 days of lactation; lane 4, brain; lane 5, heart ventricles; lane 6, thymus; lane 7, lymph nodes; lane 8, submaxillary gland; lane 9, pancreas; lane 10, sublingual gland; lane 11, tongue; lane 12, kidney; lane 13, liver; lane 14, parotid gland. Lane 16, 10 μg of RNA from the parotid gland of a male transgenic mouse. Panels A and B were exposed for 24 h except for lanes 3 and 15 of panel B, which were exposed for only 6 h.

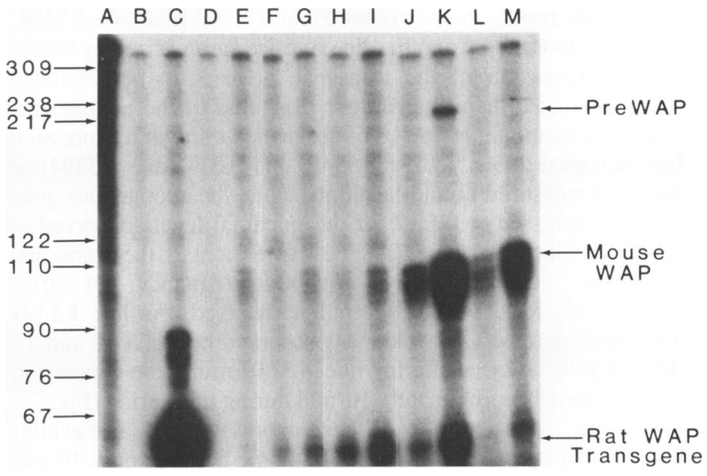

Figure 5. Developmental expression of the endogenous mouse WAP gene. Lane A, 322 NT cRNA probe for the endogenous mouse WAP first exon; lane B, yeast tRNA (10 μg); lane C, total RNA from rat mammary gland at day 13 of lactation (5 μg); lanes D-M, total RNA from the mammary gland of transgenic mice, lanes D and E, virgin (1 and 5 μg); lanes F and G, 7 days of pregnancy (1 and 5 μg); lanes H and I, 10 days of pregnancy (1 and 5 μg); lanes J and K, 17 days of pregnancy (10^{-2} and 10^{-1} mg); lanes L and M, 10 days of lactation (10^{-3} and 10^{-2} μg). Note the preWAP signal in lane K. Exposure time, 36 h.

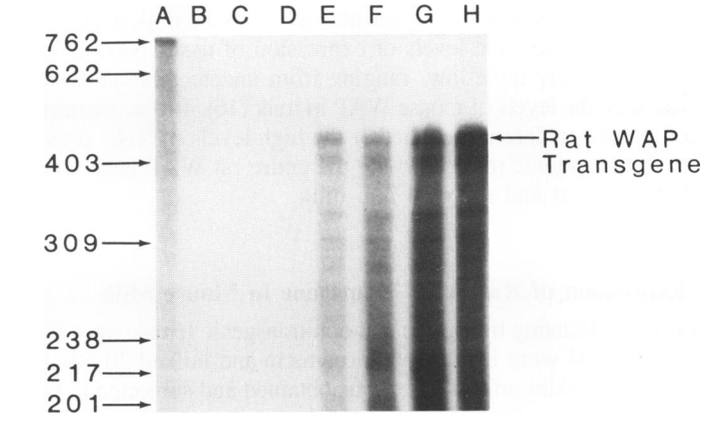

Figure 6. Developmental expression of the rat WAP gene in transgenic mice. Lane A, 762 NT rat WAP cRNA probe; lane B, yeast tRNA (10 μg); lanes C-H, total RNA isolated from the mammary gland, lane C, non-transgenic mouse at 10 days of lactation (10 μg); lanes D-H, transgenic mice, lane D, virgin (10 μg); lane E, 7 days of pregnancy (10 μg); lane F, 10 days of pregnancy (10 μg); lane G, 17 days of pregnancy (0.1 μg); lane H, 10 days of lactation (0.1 μg). 18 h exposure.

Developmental Regulation of the Rat WAP Transgene

The levels of endogenous mouse WAP and rat transgene transcripts were determined in RNA isolated from the mammary glands of mouse line #5394 at different stages of development. The same probes as described above to analyze the pattern of tissue specificity were utilized. Expression was examined in RNA isolated from mammary glands of virgin, 7, 10, and 17 day pregnant mice and compared to mice at 10 days of lactation. An obvious increase in the expression of endogenous WAP gene transcripts is not seen until day 10 of pregnancy (Fig. 5, lane I). However, increased expression of the WAP transgene was observed as early as day 7 of pregnancy (Fig. 5, lane G). A larger protected fragment of 230 NT presumably corresponding to sequences in the first intron of the mouse WAP gene has been observed previously using the same mouse WAP probe (17). This mouse 'pre-WAP' was detected also at day 17 of pregnancy (Fig. 5, lane K).

To confirm these results, the RNA samples from different stages of development during pregnancy were analyzed using the specific rat WAP cRNA probe (Fig. 6). The same kinetics of induction were observed during pregnancy as shown in Figure 5 using cross-reactivity with the mouse WAP probe. No visible expression was detected in the virgin gland; however, by 7 days of pregnancy significant expression is detected in 10 μg of total mammary gland RNA.

From the results shown in both Figures 5 and 6 for line #5394, as well as those from another line of mice summarized in Figure 7, the expression of the transgene appears to be regulated temporally in a manner different from that of the endogenous gene. For example, at day 10 of pregnancy transgene expression has increased to 9.8% (line #5349) and 0.3% (line #5394) of the level measured at mid-lactation, while the endogenous gene is only expressed at 0.03% of the maximal levels observed at day 10 of lactation (Fig.7). The expression of the transgene appears to increase steadily throughout pregnancy, but varies considerably for the two different lines assayed. For line #5394 a marked increase (267-fold) was observed between 10 and 17 days of pregnancy, while for line #5349 transgene expression only increased approximately 10-fold during this period (Fig. 7). In contrast, endogenous WAP gene expression does not exhibit a major increase (821-fold) until sometime between days 10 and 17 of pregnancy (Fig.7). The steady increase of the transgene cannot be due only to the increasing epithelial cell content of the gland during pregnancy and lactation. There is, at most, a 10–20 fold increase in glandular cells during this developmental period (32,33), while the induction levels of the transgenes between 7 days of pregnancy to mid-lactation were 100- and 1200-fold, respectively, for lines #5349 and #5394. These differences were estimated by both densitometric scanning of the appropriate areas of the gel as well as direct scintillation counting and comparison between the developmental stages.

WAP promotor constructs have been shown to direct the secretion of tissue plasminogen activator into the milk of lactating mice. However, the levels of expression of tissue plasminogen activator were quite low, ranging from undetectable to 40-fold less than the levels of mouse WAP in milk (16). It was, therefore, of interest to determine whether the high levels of RNA present in the transgenic mice carrying the entire rat WAP gene would be translated and secreted into milk.

Expression of Rat WAP Transgene in Mouse Milk

Ten day lactating transgenic and non-transgenic female mice from line #5394 were injected with oxytocin and milked 20 minutes later. The skim milk fraction was obtained and subjected to two-dimensional polyacrylamide gel electrophoresis (Fig. 8). Rat WAP contains three phosphorylation sites (34) which allows its differentiation from mouse WAP because of the more acidic pI displayed during isoelectric focusing. Although the endogenous WAP is not phosphorylated, the transgenic rat WAP exhibits the expected three phosphorylated forms of the protein (Fig. 8B), which migrated identically to authentic WAP obtained from rat skim milk (data not shown). In Figure 8, the rat WAP is readily apparent in samples isolated from the transgenic line #5394 (panel B) as compared to samples isolated from a non-transgenic mouse (panel A). Comparable results have been obtained from another line of mice, #5440 (data not shown). It is difficult to quantitate accurately the relative levels of expression in milk of the rat and mouse WAPs in transgenic and non-transgenic lines by Coomassie blue staining; however, the high levels of the transgenic rat WAP expressed in milk did not appear to inhibit the relative expression of the mouse WAP. Quantitative RNase protection assays comparing the expression in transgenic and non-transgenic paired littermates also support the lack of inhibition of endogenous mouse WAP gene expression even in the highest expressing lines bearing the rat WAP transgene (data not shown).

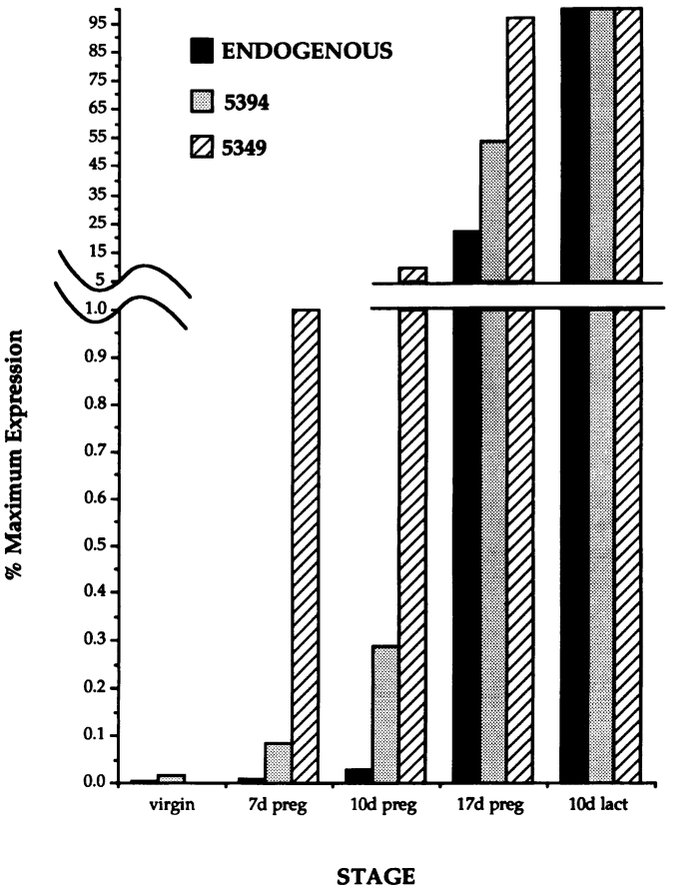

Figure 7. Developmental induction of endogenous and transgenic WAP expression. The expression levels from virgin, 7, 10, and 17 days of pregnancy and at 10 days lactation in transgenic mice were quantitated as described in text. The percentage of 10 day lactating levels at each stage for both the endogenous mouse WAP and the transgenes were determined and compared. The induction is expressed as the% of 10 day lactating expression for each developmental stage.

DISCUSSION

The efficient expression of the 4.3 kb rat WAP construct containing only 0.949 kb of 5′ flanking DNA in transgenic mice was unexpected, considering the relatively inefficient expression of WAP promoter-reporter gene constructs containing 2.5 kb of 5′ flanking DNA (16). Both constructs elicit tissue-specific and developmentally controlled expression of the transgenes, and secretion of WAP or a targeted heterologous gene into the milk of transgenic mice. In both cases, the temporal expression of these constructs also was altered as compared to the pattern of expression observed for the endogenous WAP gene. The transgene is upregulated sooner in pregnancy than the endogenous gene, even though its level of expression during lactation is less than the endogenous gene. The increase in WAP transgene expression observed between days 7 and 10 of pregnancy is reminiscent of the pattern of casein gene expression seen during this developmental period, a time during which a marked increase occurs in serum placental lactogen (35). In explant cultures isolated from mammary glands of 14 day pregnant rats, both WAP and casein gene expression are regulated by glucocorticoids and prolactin, but with differing sensitivities to these steroid and peptide hormones (9). However, a marked increase in endogenous WAP gene expression is not observed until day 16

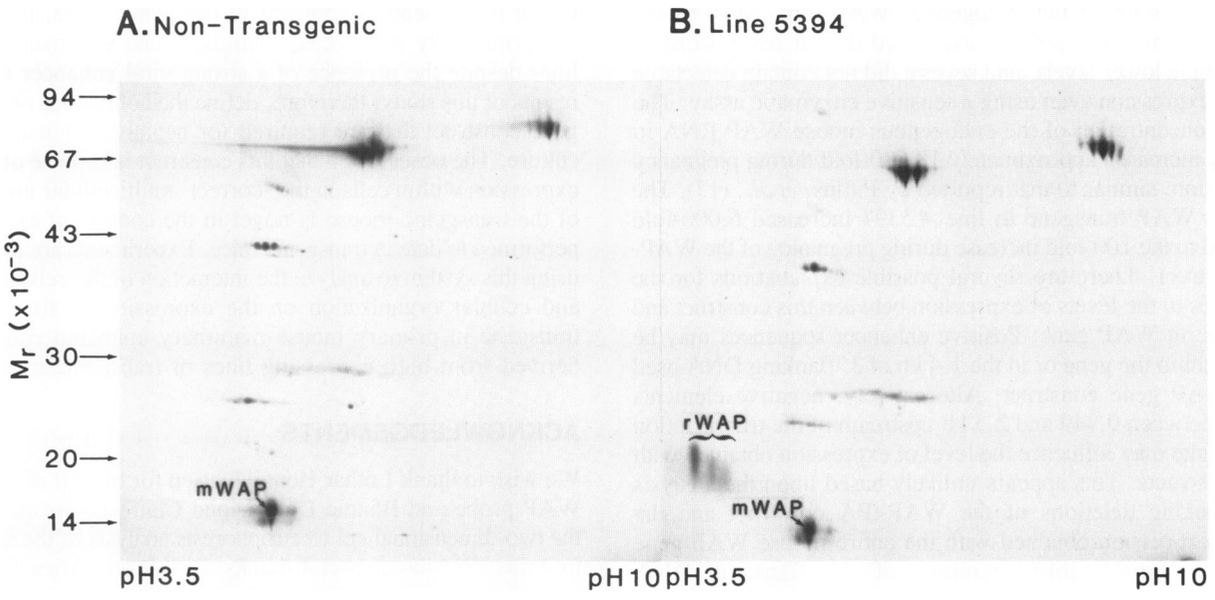

Figure 8. Two-dimensional polyacryamide gel electrophoresis of non-transgenic (A) and rat WAP transgenic mouse skim milk. Approximately 80 μg of skim milk proteins for each gel was subjected to isoelectric focusing in the first dimension and SDS-polyacrylamide sizing through 10−20% gradient gels in the second dimension (acidic end on the left and basic end on the right). The gel was stained with Coomassie Blue to visualize the proteins. Labelled arrows indicate the endogenous WAP protein and three phosphorylated forms of the rat WAP transgenic protein. Other proteins found in the skim milk are from decreasing molecular weights: transferrin (80 kD), albumin (68 kD), α1 (44 kD) and α2-casein (41 kD), β-casein (26 kD), γ-casein (24 kD), and δ-casein (20 kD). The protein spot directly under mouse WAP at 14 kD is α-lactalbumin (23).

of pregnancy, considerably after the increase in the serum levels of lactogenic hormones (17). This restriction in the timing of expression of the endogenous mouse WAP gene is not observed for transgene expression in mice bearing either the 4.3 kb rat WAP or the 2.5 kb mouse WAP promoter gene constructs. Conceivably, sequences important for this restriction may be missing from both of these constructs. Alternatively, developmentally regulated changes in the higher order structure of the WAP gene perhaps requiring a unique chromosomal location, may be required to allow the appropriate hormonal control of WAP gene expression during mammary differentiation. For example, mouse line, #5349, whose transgene is expressed at a 10-fold lower level at day 10 of lactation than the endogenous WAP gene, reaches 97% of its maximum expression level by day 17 of pregnancy. In contrast, line #5394 displayed a 2-fold increase in expression during this developmental period. Thus, even the precise temporal control of transgene expression appears to be dependent on chromosomal location.

The high level of expression obtained with the entire rat WAP gene is analogous to results seen in transgenic mice carrying the entire sheep β-lactoglobulin gene (36). β-lactoglobulin is the major whey protein of ruminants and not found in rodents. However, in transgenic mice containing the entire β-lactoglobulin gene with 4 kb of 5′ and 1.6 kb of 3′ sequence, high levels, approaching those observed in sheep, of β-lactoglobulin mRNA were observed in mice during lactation. The sheep β-lactoglobulin gene exhibited mammary-specific expression and, interestingly, was once again developmentally regulated temporally similar to the endogenous mouse β-casein gene (36, P. Simons and J. Clark, personal communication). Analogous to the rat WAP transgenics, several lines of mice bearing the β-lactoglobulin gene secreted large amounts of the transgene encoded protein into milk (36). Efficient expression of the entire mouse WAP gene also has been obtained recently in transgenic pigs (R. Wall and L. Hennighausen, personal communication). Thus, the cis-acting sequences and trans-acting factors required to regulate both β-lactoglobulin and WAP gene expression appear to be reasonably well conserved even in other mammalian species not normally expressing these whey protein genes.

These results can be contrasted with the inefficient expression (<1% of the endogenous level) of the entire rat β-casein gene containing 3.5 kb of 5′ and 3.0 kb of 3′ flanking DNA in transgenic mice (18). Like WAP, β-lactoglobulin exists as a single copy gene perhaps indicating enhancer proximity not seen in genes such as β-casein, which is a member of a gene family present as a gene cluster on mouse chromosome 5 (37). At present it is unclear whether the intrinsic differences in the expression of the casein and whey protein genes in transgenic mice reflect the location of a mammary-specific enhancer in the immediate vicinity of the whey protein constructs. One method for identifying such an enhancer will be to test its effect on the expression of chimeric rat β-casein constructs in transgenic mice.

Dominant control regions specifying copy-number dependent and tissue-specific levels of gene expression independent of the site of integration have been found at relatively great distances from the β-globin gene, a member of the developmentally regulated globin multigene family (38). While the expression of the WAP and β-lactoglobulin genes is relatively efficient in transgenic mice, these transgenes still did not display copy-number dependent or position-independent expression. An additional example of this type of transgene expression has been reported recently using another, single copy whey protein gene encoding bovine α-lactalbumin (39). Thus, analogous dominant control regions surrounding any of the milk protein genes have yet to be identified.

In contrast to the high levels of expression seen with the entire WAP gene, the highest level of expression observed using WAP-tPA constructs containing 2.5 kb of 5′ flanking sequences, was

only 1% the level of the endogenous WAP (16). Most of the other transgenic lines assayed expressed tPA at several orders of magnitude lower levels, and several did not contain detectable levels of expression even using a sensitive enzymatic assay. The relative concentration of the endogenous mouse WAP RNA in this study increased approximately 18,000-fold during pregnancy and lactation, similar to that reported by Pittius, *et al.*, (17). The entire rat WAP transgene in line #5394 increased 6,000-fold compared to the 100-fold increase during pregnancy of the WAP-tPA construct. There are several possible explanations for the differences in the levels of expression between this construct and the entire rat WAP gene: Positive enhancer sequences may be present within the gene or in the 1.4 kb of 3′ flanking DNA used in the WAP gene construct. Alternatively, negative elements residing between 0.949 and 2.5 kb upstream of the transcription initiation site may influence the level of expression obtained with these constructs. This appears unlikely based upon the analysis of 5′ flanking deletions of the WAP-tPA construct and the efficient expression obtained with the entire mouse WAP gene containing comparable amounts of 5′ flanking DNA (L. Hennighausen and T. Burdon, personal communication).

The relatively inefficient nature of the WAP-tPA construct also appears to be a reflection of the tPA cDNA reporter gene construct, which may have lacked the appropriate exon/intron structure for efficient expression in transgenic mice (31). Differences in both the penetrance and expression of constructs in transgenic mice have been observed using the same promoter/enhancer fragment linked to either cDNA or genomic DNA reporter gene constructs (40,41). It has been reported that the inclusion of the genomic sequences containing introns may influence the efficiency of expression in transgenic mice through enhanced transcription and not by affecting RNA processing (31). However, recent studies in which the mouse WAP promotor fused to the entire gene encoding the breast cancer protein, PS2, in 7 lines of transgenic mice was not expressed (38). Therefore, a heterologous promotor alone even with a genomic reporter gene may not be sufficient for expression. In the same report, a heterologous, 490 NT PS2 cDNA inserted into the first exon of the mouse WAP gene resulted in the expression of the hybrid transcript at only 2.5% the level of the endogenous WAP transcript and in only one line of transgenic mice carrying 20 copies of the transgene (40). Thus, in this case even though the entire mouse WAP gene, *i.e.* a 7.5 kb *Eco*RI fragment containing 2.5 kb of 5′ flanking DNA, was used to generate transgenic mice, the nature of the construction resulted in inefficient expression.

Finally, there is evidence that the regulation of WAP mRNA accumulation occurs at the post-transcriptional level rather than solely through increased transcription (7,10, R., McKnight and L. Hennighausen). Therefore, the presence of sequences within the gene or in the 3′ flanking DNA may be necessary to facilitate post-transcriptional processing either in the nucleus or at the level of cytoplasmic mRNA stability.

Recently, a culture system utilizing extracellular matrix has been devised that allows expression of WAP in primary mouse mammary epithelial cells only when these cells organize into 3-dimensional alveolar-like spherical structures surrounding a closed lumen (10). In the absence of a multicellular organization of mammary cells into the correct 3-dimensional structure, WAP gene expression is inhibited. Furthermore, nuclear run-on experiments have suggested that this suppression of WAP gene expression is post-transcriptional. These observations may explain the failure to observe expression of the 4.3 kb rat WAP construct, which is efficiently expressed in transgenic mice, in a variety of permanently transfected mammary and non-mammary cell lines despite the presence of a strong viral enhancer (12). The results of this study, therefore, define the boundaries of the WAP gene construct that are required for negative regulation in cell culture. The observation that this construct is capable of efficient expression within cells in the 'correct' multicellular architecture of the transgenic mouse is novel in the context of experiments performed to date in transgenic mice. Experiments are in progress using this system to analyze the interaction of the cell substratum and cellular organization on the expression of the rat WAP transgene in primary mouse mammary epithelial cell cultures derived from high expressing lines of transgenic mice.

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