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Biochemistry

A milk protein gene promoter directs the expression of human tissue plasminogen activator cDNA to the mammary gland in transgenic mice

(whey acidic protein/mouse mammary gland/lactation/hormone regulation/tissue-specific expression)

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ABSTRACT **Whey acidic protein (WAP) is a major whey protein in mouse milk. Its gene is expressed in the lactating mammary gland and is inducible by steroid and peptide hormones. A series of transgenic mice containing a hybrid gene in which human tissue plasminogen activator (tPA) cDNA is under the control of the murine WAP gene promoter had previously been generated. In this study, 21 tissues from lactating and virgin transgenic female mice containing the WAP-tPA hybrid gene were screened for the distribution of murine WAP and human tPA transcripts. Like the endogenous WAP RNA, WAP-tPA RNA was expressed predominantly in mammary gland tissue and appeared to be inducible by lactation. Whereas WAP transcripts were not detected in 22 tissues of virgin mice, low levels of WAP-tPA RNA, which were not modulated during lactation, were found in tongue, kidney, and sublingual gland. These studies demonstrate that the WAP gene promoter can target the expression of a transgene to the mammary gland and that this expression is inducible during lactation.**

Synthesis and secretion of many milk proteins is specific to the lactating mammary gland and expression of the corresponding genes is modulated by steroid and peptide hormones (1). Our goal is to identify and characterize control elements governing milk protein gene expression. Although cell culture systems have been used to define DNA-regulatory elements such as promoters, enhancers, and hormone-responsive elements in a number of genes, hormone-regulated and tissue-specific expression of milk protein genes has not yet been demonstrated in cell lines. The transgenic mouse appears to be an appropriate system for the characterization of gene-regulatory elements within the context of the organism (2–8).

The whey acidic protein (WAP) gene, encoding a major murine milk protein (9), has been cloned (10, 11) and putative regulatory elements in the promoter region have been identified (12). We have previously described the generation of transgenic mice carrying hybrid genes containing the murine WAP gene promoter and the human Ha-*ras* gene (13) or human tissue plasminogen activator (tPA) (14) cDNA. Human tPA is a protease capable of fibrinolysis and has been shown to have efficacy in the treatment of clotting disorders. Aside from providing information about WAP gene regulation, the transgenic mice carrying the WAP-tPA hybrid gene represent a model system for the cost-effective production of human therapeutic proteins in milk.

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In this report, we identify transgenic mice carrying the WAP-tPA hybrid gene, analyze the authenticity of tPA product secreted into milk, and compare the steady-state levels of WAP and WAP-tPA RNAs in several tissues from lactating and virgin transgenic animals.

MATERIALS AND METHODS

Production of Transgenic Mice. The hybrid gene WAP-tPA, consisting of the promoter region of the WAP gene (−2600 to +24) fused to the cDNA coding for human tPA, was introduced into mouse embryos as a *Hin*dIII–*Bam*HI fragment (14). DNA was microinjected into one-cell fertilized eggs and transgenic mice were derived as described (14).

Isolation of DNA and RNA. A short segment of mouse tails was removed and genomic DNA was prepared by the procedure of Hogan *et al.* (15). RNA was isolated by extraction with acid guanidinium isothiocyanate/phenol/chloroform (16). The integrity of RNA was confirmed on formaldehyde gels stained with ethidium bromide. Poly(A) RNA was prepared by chromatography on oligo(dT)-cellulose (Pharmacia; ref. 17).

DNA Analysis. Mice carrying the hybrid gene were identified by Southern blot analysis (18) of *Eco*RI-digested DNA extracted from tails using a probe specific for the promoter of the WAP gene. The probe consisted of a 1700-base-pair (bp) *Bgl* II–*Kpn* I fragment (−1700 to +24) of the WAP gene (11) labeled with ^{32}P by using random hexanucleotide primers (19).

Preparation of Antisense RNA Probes. A 641-bp *Hin*fI fragment from the WAP gene (−413 to +228, see ref. 11) was blunt ended with mung bean nuclease and cloned into the polylinker of the Bluescribe vector pBS (Stratagene, La Jolla, CA). After linearizing the plasmid at the *Xba* I site (nucleotide −88 in the WAP gene promoter), antisense RNA labeled with ^{32}P to a specific activity of 10^9 cpm/μg was produced with T7 RNA polymerase. For detection of WAP-tPA hybrid RNA, the probe was synthesized with T7 RNA polymerase from a plasmid containing the 220-bp *Xba* I–*Bgl* II fragment that spans the WAP-tPA junction (see Fig. 3).

RNase Analysis. Ten micrograms of total RNA isolated from tissue was incubated with 200,000 cpm of ^{32}P-labeled antisense RNA in 30 μl of 40 mM Pipes, pH 6.7/0.4 M NaCl/80% formamide as described (20). After denaturation at 85°C for 5 min, tubes were incubated at 37°C for 12 hr. RNase digestion was performed by adding 300 μl of 0.3 M NaCl/10 mM Tris·HCl, pH 7.5/5 mM EDTA/40 μg of RNase

Abbreviations: WAP, whey acidic protein; tPA, tissue plasminogen activator; MMTV, mouse mammary tumor virus; LTR, long terminal repeat.

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A per ml (Sigma)/10 μg of RNase T1 per ml followed by incubation for 15 min at 30°C. After phenol/chloroform extraction, the RNA was precipitated with ethanol and digested with 2 units of RNase H (Bethesda Research Laboratories) in 20 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/100 mM KCl for 20 min at 37°C. Following digestion with proteinase K for 15 min at 37°C, extraction with phenol/chloroform, and ethanol precipitation, RNA was dissolved in 80% formamide and analyzed by electrophoresis in a 6% polyacrylamide/8 M urea gel. The gels were dried on Whatman paper and subjected to autoradiography at −70°C with intensifying screens for 12–150 hr. We calculate that this assay was sufficiently sensitive to detect one molecule of WAP mRNA per cell.

tPA Assays. Assays were performed with an ELISA kit (Immunobind). The standard curves were performed in negative mouse milk diluted to a final concentration of 10% with phosphate-buffered saline, to which was added tPA supplied with the kit. All points of the control curve and the experimental assays have the background value (the value determined for negative mouse milk) subtracted. Values were confirmed by using a fibrin clot lysis assay (14) (data not shown).

Electrophoretic Transfer Blot. Samples of the whey fraction from milk, soluble material following acid precipitation of milk, along with purified Bowes melanoma tPA were run on a 10–20% $NaDodSO_4$/polyacrylamide gradient gel according to the method of Laemmli (21). The proteins were electrophoretically transferred to nitrocellulose and incubated with goat anti-human melanoma tPA (American Diagnostica, Greenwich, CT). The detection system involved a horseradish peroxidase-conjugated rabbit anti-goat IgG and was developed with 4-chloro-1-naphthol (Bio-Rad).

RESULTS

Expression of Human tPA in Mouse Milk. A hybrid gene consisting of 2.6 kilobases (kb) of upstream DNA from the WAP gene and the cDNA encoding human tPA (14) was introduced into mouse oocytes. The WAP portion of this fusion gene terminates in the 5′ untranslated region within the first exon of the WAP gene, 24 bp downstream of the transcriptional start site. The tPA part of the WAP-tPA hybrid gene contains the start site of translation and the native secretion signal sequence from tPA. Six transgenic mouse lines were identified containing the WAP-tPA hybrid gene. Some preliminary data on three of these lines have been presented (14).

The copy number of integrated WAP-tPA sequences varied between 3 and 50 in these lines (Fig. 1*A*). Tail DNA was digested with *Eco*RI, which cleaves 5′ of the WAP promoter and twice within the tPA gene. The DNA was separated in an agarose gel, transferred to a filter, and probed with 5′ flanking DNA of the WAP gene, thereby permitting detection of a WAP-tPA gene fragment and the endogenous WAP gene. The 1.7-kb WAP gene probe consistently hybridized to two *Eco*RI fragments. One is a 6.5-kb fragment indicative of the endogenous WAP gene, which served as an internal hybridization standard for a single-copy gene; the other is a 3.3-kb fragment from the WAP-tPA hybrid gene. Most of the hybrid genes appeared to be integrated as multiple head-to-tail copies and were inherited in Mendelian fashion (data not shown).

The WAP-tPA construction was designed to facilitate expression in lactating mammary epithelial cells and to allow secretion of the gene product, human tPA, into milk. Transgenic female mice from each line were mated in order to propagate the strains and to provide milk samples for analysis. Human tPA was immunologically detected in the milk of mice during midlactation in four of the six lines (Fig. 1*C*). In separate experiments, using an *in vitro* clot lysis assay (ref. 22; data not shown), the tPA from the milk of these transgenic animals was biologically active. To correlate the amount of tPA in the milk from the different lines with the expression of the hybrid gene, poly(A) RNA was prepared from lactating mammary glands and analyzed in an RNase protection assay. The amount of tPA in the milk of the different lines correlated with the relative concentrations of WAP-tPA hybrid RNA (Fig. 1 *B* and *C*). However, there was no direct correlation between the copy number of WAP-tPA genes and the level of tPA expression (Fig. 1). In fact, line 27 produced the highest amounts of human tPA in the milk but contained the smallest number of WAP-tPA gene copies. The amount of tPA found in milk of the line 27 was ≈50 μg/ml of milk as judged by an ELISA.

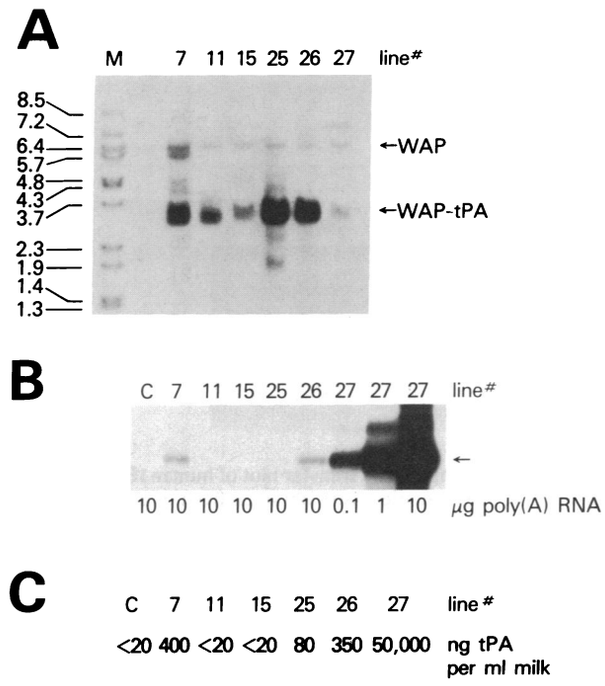

FIG. 1. (*A*) Southern blot analysis of genomic DNA from one member of each of the six transgenic lines. Tail DNA, 10 μg, from each animal was restricted with *Eco*RI and electrophoresed on a 0.8% agarose gel, transferred to a nitrocellulose filter, and hybridized with a probe of the WAP gene promoter. By using this probe the endogenous WAP gene (6.5-kb band) and a 3.3-kb fragment indicative of the WAP-tPA hybrid gene are visualized. The 3.3-kb fragment contains a 2.6-kb WAP gene promoter sequence and 700-bp tPA cDNA sequence. As can be estimated from the intensity of the 3.3-kb fragment, the copy number of the integrated transgene varied considerably between different lines. The size marker (lane M; sizes in kb) is a ^{32}P-labeled *Bst*EII digest of λ DNA. (*B*) RNase protection analysis of poly(A) RNA prepared from lactating mammary gland of mice from each of the six lines and nontransgenic control mice (lane C). The arrow indicates the WAP-tPA-specific transcript. The slightly larger transcript seen in line 27 might point to a second upstream start site used in a small percentage of the transcripts. (*C*) Average tPA concentration in the milk of lactating animals from the six lines and control mice (lane C), as determined independently by ELISA and clot lysis assay.

Fig. 2 shows an electrophoretic transfer blot characterizing the human tPA in milk of line 27 (Fig. 2, lane b). Several unique bands are evident in transgenic mouse milk as compared to the milk taken from a nontransgenic mouse (Fig. 2, lane a). For comparison, human tPA from Bowes melanoma (Fig. 2, lane c) consists of two predominant forms: single-chain material (64–65 kDa) and the more rapidly migrating two-chain form (30–36 kDa). The predominant band of the samples obtained from milk of line 27 after electrophoresis had a mobility similar to that of the two-chain

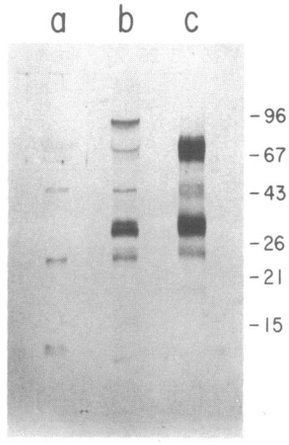

FIG. 2. Electrophoretic transfer blot of human tPA secreted into mouse milk. Milk samples were obtained from mice of line 27 (lane b) and from lactation-stage and age-matched nontransgenic control mice (lane a) by treatment of the mothers with oxytocin and manual manipulation as described (14). The whey fraction was prepared by removing the caseins with an acid treatment and centrifugation. The soluble (whey) fractions were neutralized and run on a polyacrylamide gel, and the human tPA samples were stained. Lane c, positive control from a Bowes melanoma cell line. Protein size markers are indicated (in kDa) on the right.

form of Bowes melanoma tPA (Fig. 2). Another protein band in milk of line 27 correlated well in size with the single-chain form, although the patterns are distorted by an excess of whey proteins in that molecular mass range (Fig. 2, lane b). The immunostained background pattern of the normal whey control may be due to crossreacting endogenous mouse tPA or nonspecific binding of whey proteins to the human polyclonal antibody. The presence of a higher molecular mass form of reactive protein than the single-chain form of tPA may represent a complex formed with a tPA inhibitor or an aggregate.

Tissue-Specific Expression of the WAP and WAP-tPA Gene. To assess tissue-specific expression of the endogenous WAP and WAP-tPA hybrid gene in line 27 we analyzed RNA from 21 tissues of lactating females by using an RNase protection assay. The WAP-specific template (Fig. 3*A*) consisted of a 322-bp fragment from nucleotide −88 to +234 within the first intron of the WAP gene. RNA synthesized *in vitro* from this template was hybridized with mammary gland RNA and digested with RNase. As predicted, a 112-bp fragment was generated, corresponding to the size of the first exon (Fig. 4*A*). Fig. 3*B* shows the corresponding WAP-tPA template. Upon hybridization of a probe derived from this template

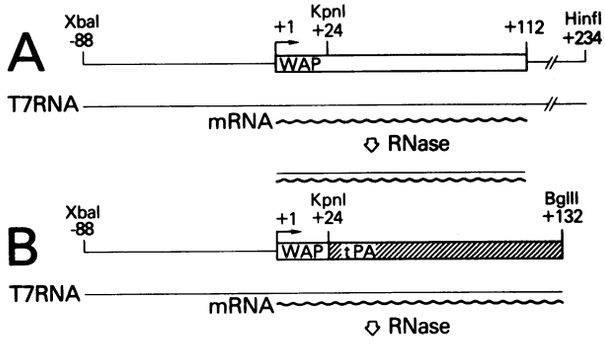

FIG. 3. Templates used for RNase mapping. The subcloned fragments used for antisense T7 polymerase transcription and generation of the WAP-specific RNA (*A*) and the WAP-tPA-specific RNA (*B*) are shown in the top portions. The predicted fragments that are protected from digestion with RNase are indicated by wavy lines.

with mammary gland RNA and RNase digestion, a 132-bp fragment indicative of the WAP-tPA hybrid transcript remained protected (Fig. 4*B*).

Steady-state levels of the two types of transcript were determined with the WAP- and WAP-tPA-specific probes in 21 tissues of lactating females of line 27. This line was chosen because it produced the highest levels of WAP-tPA RNA and human tPA protein. The major site of WAP and WAP-tPA RNA accumulation was the mammary gland (Fig. 4). The steady-state level of endogenous WAP RNA in the lactating mammary gland is ≈100-fold higher than that of WAP-tPA RNA. Lower levels of WAP RNA (by a factor of $\approx 10^4$–10^6) were detected in pituitary gland, pancreas, adrenal gland, tongue, liver, thymus, and heart atrium (Fig. 4*A*). WAP-tPA RNA at levels lower than in the lactating mammary gland (by a factor of at least 100) were detected in tongue, sublingual gland, and kidney (Fig. 4*B*). These results were confirmed by using sibling animals (data not shown).

Hormonal Regulation of WAP and WAP-tPA Gene Expression. Expression of the WAP gene in the mammary gland appears to be under the control of hormones (1). To assess whether control elements within the WAP-tPA hybrid gene were sufficient to confer hormone regulation, we analyzed 22 tissues of 6-week-old virgin females from line 27 for the presence of WAP and WAP-tPA RNA. No WAP-specific transcripts were detected in 10 μg of total RNA from each of the 22 tissues (Fig. 5*A*). In contrast, WAP-tPA RNA was found in the mammary gland, sublingual gland, tongue, and kidney of virgin females (Fig. 5*B*). Whereas the level of WAP-tPA RNA in the lactating mammary gland was about 100-fold higher than in virgin mammary glands, the levels of WAP-tPA RNA in kidney, sublingual gland, and tongue appeared to be unaffected by lactation. Since the relative number of mammary epithelial cells increases only about 5-fold during pregnancy (23), the 100-fold increase of WAP-tPA RNA is likely to reflect significant hormone regulation of the hybrid gene.

DISCUSSION

The WAP-tPA hybrid gene was stably integrated in six lines of transgenic mice. Active human tPA was found in milk obtained from mice of four lines and WAP-tPA hybrid RNA was found predominantly in the lactating mammary gland. These observations suggest that regulatory elements targeting expression to the lactating mammary gland are located within 2500 bp of WAP gene upstream sequence.

The level of active tPA in the milk of different lines varied over 3 orders of magnitude, with one line accumulating 50 μg/ml, or about 5% of the level of endogenous WAP. The varying amounts of human tPA in the milk correlated with the concentrations of WAP-tPA hybrid RNA in the lactating mammary glands of these mice, suggesting that differential secretion of tPA into the milk was not responsible for these differences. Also, no correlation could be established between the level of tPA expression and the copy number of WAP-tPA genes. This is in agreement with studies on several different genes (5). Rather, it appears likely that variability in the expression of the WAP-tPA gene may be due to position effects caused by the random insertion of the hybrid gene into the genome. This is corroborated by the variable expression pattern of another hybrid gene using the WAP gene promoter in transgenic mice, the WAP/Ha-*ras* oncogene construct (13).

Human tPA secreted into milk was verified to be authentic by electrophoretic transfer blot analysis using an antibody specific for human tPA. In contrast to the molecular form of the Bowes melanoma standard, recombinant tPA produced in mouse milk appeared to be primarily of the two-chain form. The predominance of a two-chain material could be due to endogenous plasmin present in milk. The material is biolog-

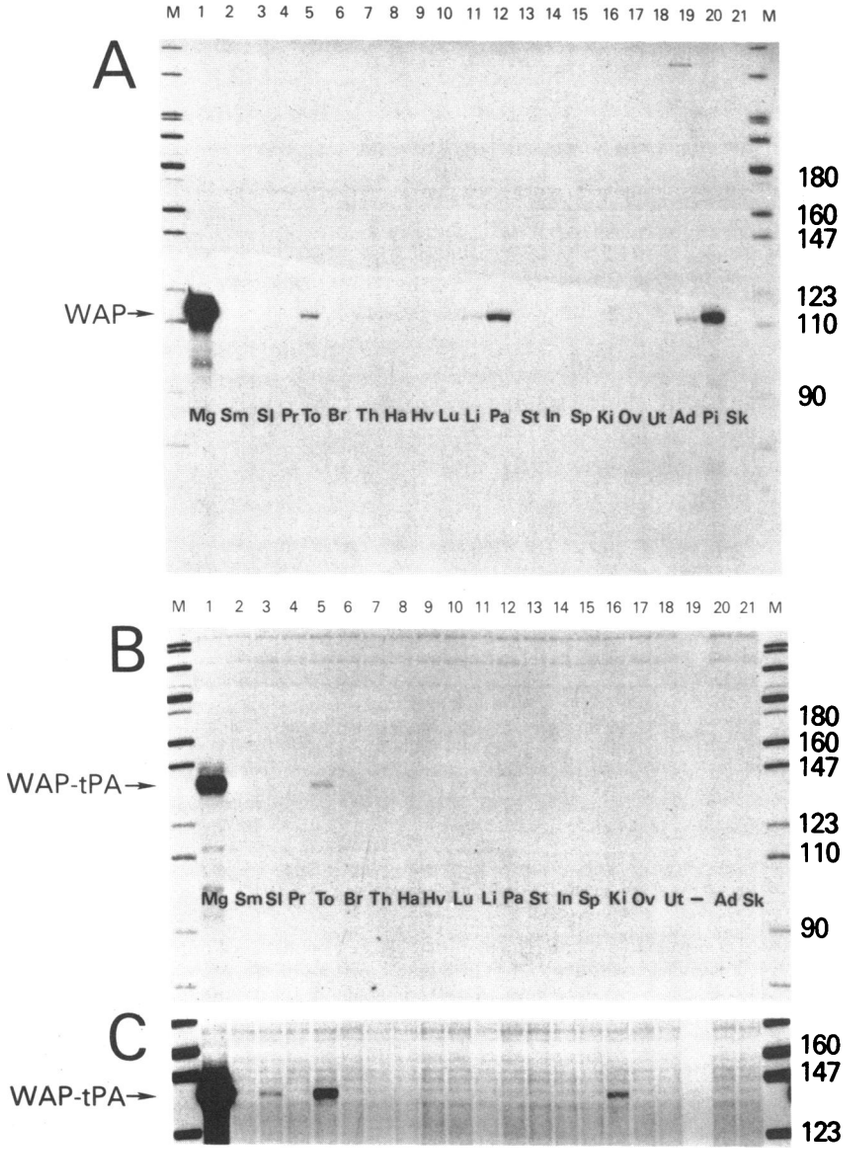

FIG. 4. Tissue distribution of endogenous WAP RNA (*A*) and WAP-tPA hybrid RNA (*B* and *C*) in a lactating mouse of line 27. RNA from 21 tissues was hybridized with radioactively labeled T7 RNA complementary to the WAP gene (*A*) or the WAP-tPA hybrid gene (*B* and *C*) and then digested with RNase; the reaction products were separated in a sequencing gel. The arrows indicate the protected 112-bp fragment encoding the first exon of endogenous WAP gene (*A*) and the 132-bp fragment indicative of the WAP-tPA hybrid RNA (*B* and *C*). The size marker (lanes M; sizes in bp) is a ^{32}P-labeled *Msp* I digest of pBR322. In lane 1 of *A*, 0.1 μg of total RNA from lactating mammary glands (Mg) was analyzed. All other lanes represent the hybridization signal upon incubation with 10 μg of total RNA. Sm, submaxillary gland; Sl, sublingual gland; Pr, parotid gland; To, tongue; Br, brain; Th, thymus; Ha, heart atrium; Hv, heart ventricle; Lu, lung; Li, liver; Pa, pancreas; St, stomach; In, intestine; Sp, spleen; Ki, kidney; Ov, ovary; Ut, uterus; Ad, adrenal gland; Pi, pituitary; Sk, skin. (*A* and *B*) Twelve-hour exposures. (*C*) Four-day exposure of *B*.

ically active, as judged by clot lysis assay; however, the exact specific activity of the human tPA produced in milk remains to be determined.

From our RNase protection studies it appears that the transcriptional start sites in the WAP-tPA and WAP genes are identical. Interestingly, in the WAP/Ha-*ras* transgenic mice a cryptic start site 3′ to the native one was utilized (13), suggesting that selection of transcriptional start sites may be influenced by sequences fused to the WAP gene promoter. Although the WAP-tPA gene is expressed predominantly in the lactating mammary gland, its expression pattern does not tightly follow that of the endogenous WAP gene; though no WAP RNA was found in any of 22 tissues from virgin females, low levels of WAP-tPA RNA were detected in mammary gland, sublingual gland, tongue, and kidney. The level of WAP-tPA transcripts in the mammary gland was greatly elevated during lactation, but the concentrations in sublingual gland, tongue, and kidney did not change significantly, suggesting that hormonal control of WAP-tPA gene expression is confined to the mammary gland. This notion is further supported by the presence of low amounts of WAP-tPA transcripts in sublingual gland, tongue, and kidney in transgenic males (data not shown).

The extent of hormonal induction and the absolute level of expression of the WAP-tPA gene are low compared to the endogenous WAP gene, suggesting that important cis-acting elements may be missing from the WAP gene promoter or may fail to function in this construct. Alternatively, the chromosomal environment and/or the sequence composition of the hybrid gene could affect the expression pattern as previously discussed in a different context (24). Complementary results were obtained with another well-studied mammary-specific expression system, the murine mammary tumor virus (MMTV). Endogenous MMTV sequences are transcribed preferentially in the mammary glands of lactating animals and critical control elements within the long terminal repeat (LTR) have been identified. However, studies with transgenic animals carrying the MMTV LTR fused to a variety of reporter genes revealed that the LTR can be expressed in at least 12 different organs (7, 8). In some cases, like spleen and salivary gland, transcription from the MMTV LTR was as high as in mammary tissue (7). Thus it appears that transcription of the endogenous WAP gene, and even more pronounced transcription of the MMTV LTR, in only lactating mammary epithelial cells must require other factors or sequences in addition to the promoter regions to achieve this higher order of specificity.

A detailed knowledge of mammary and hormone-regulated milk protein gene expression provides the basis for a biotechnology aimed at producing large amounts of complex proteins. Currently, macromolecular human pharmaceuticals—e.g., tPA and blood-clotting factors VIII and IX—are

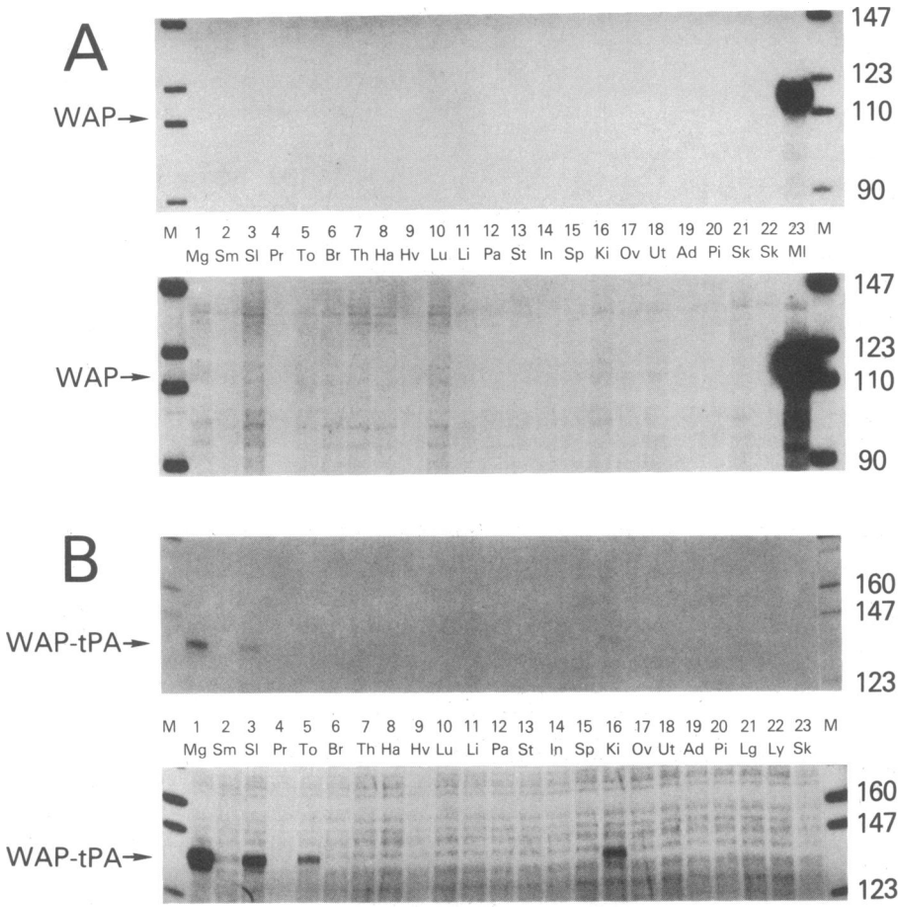

FIG. 5. Tissue distribution of WAP and WAP-tPA RNAs in a 6-week-old virgin female of line 27. Ten micrograms of total RNA from 22 tissues was analyzed for WAP and WAP-tPA RNA as described in the legend to Fig. 4. (*A*) Expression pattern of WAP RNA (12-hr and 4-day exposures). (*B*) Expression pattern of WAP-tPA hybrid RNA (24-hr and 5-day exposures). Lg, lacrimal gland; Ly, lymph node. See legend to Fig. 4 for other tissue abbreviations. In *A*, lane 23 (Ml), 0.1 μg of lactating mouse mammary gland RNA of line 27 was analyzed as a control. The size marker (lanes M; sizes in bp) is a ^{32}P-labeled *Msp* I digest of pBR322.

isolated from natural sources or are produced in tissue culture. Production of foreign proteins in the milk of transgenic dairy animals appears to be an alternative and cost-effective means for isolating human pharmaceuticals and other proteins in large quantities (25, 26). By using a mouse model system, we have succeeded in targeting the expression of active human tPA to the lactating mammary gland (ref. 14; this paper). Optimization of the mammary expression system and a transfer to dairy animals are dependent on further advances in our understanding of the molecular basis of regulated gene expression in the lactating mammary gland.

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