Expression of a Whey Acidic Protein Transgene during Mammary Development

EVIDENCE FOR DIFFERENT MECHANISMS OF REGULATION DURING PREGNANCY AND LACTATION*

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Expression of the mouse whey acidic protein (WAP) gene is specific to the mammary gland, is induced several thousand-fold during pregnancy, and is under the control of steroid and peptide hormones. To study developmental regulation of the mouse WAP gene, a 7.2-kilobase (kb) WAP transgene, including 2.6 kb of 5'- and 1.6 kb of 3'-flanking sequences, was introduced into mice. Of the 13 lines of mice examined, 6 expressed the transgenes during lactation at levels between 3 and 54% of the endogenous gene. Although expression was dependent on the site of integration, the transgenes within a given locus were expressed in a copy number-dependent manner and were coordinately regulated. The WAP transgenes were expressed specifically in the mammary gland, but showed a deregulated pattern of expression during mammary development. In all six lines of mice, induction of the WAP transgenes during pregnancy preceded that of the endogenous gene. During lactation, expression in two lines increased coordinately with the endogenous gene, and in three other lines of mice, transgene expression decreased to a basal level. These data indicate that the 7.2-kb gene contains some but not all of the elements necessary for correct developmental regulation. At a functional level it appears as if a repressor element, which inactivates the endogenous gene until late pregnancy, and an element necessary for induction during lactation are absent from the transgene. Complementary results from developmental and hormone induction studies suggest that WAP gene expression during pregnancy and lactation is mediated by different mechanisms.

Expression of milk protein genes is dependent upon interactions between tissue-specific and developmentally and hormonally induced regulatory factors (1). Our laboratory uses the mouse whey acidic protein (WAP) gene as a model to study the structure and function of the corresponding regulatory elements. The WAP gene encodes the major whey protein in mice (2), rats (1), and rabbits (3) and is expressed almost exclusively in the mammary gland. The steady state level of mouse WAP mRNA increases several thousand-fold between the virgin state and mid-lactation (4, 5). This induction depends upon the presence of lactogenic hormones, glucocorticoids, and insulin (5) but may also require some, as yet undefined, features of cell-cell interactions occurring within the mammary gland (6).

WAP gene expression increases sharply between day 15 and 17 of pregnancy (5), a period during which the levels of placental lactogens are near maximal but prolactin levels are low (7). Insulin and hydrocortisone are present throughout pregnancy. In contrast to WAP the β-casein gene is induced at day 10 of pregnancy (8), coincident with the increase in placental lactogens (7). In spite of their different temporal patterns of expression during pregnancy, induction of both the WAP and β-casein genes in organ explant cultures from mid-pregnant mice requires all three hormones, insulin, hydrocortisone, and prolactin (5, 8–10). The mechanisms by which these hormones activate milk protein gene expression is unclear. Doppler et al. (11) have shown in tissue culture cells transfected with a β-casein gene that prolactin and hydrocortisone act through promoter sequences and therefore presumably on the transcriptional level. However, Rosen and co-workers (12) have evidence that these hormones act predominantly at a post-transcriptional level.

Previous studies have shown that a hybrid gene containing 5'-flanking sequences of the mouse WAP gene is expressed specifically in the mammary gland of transgenic mice (4, 13), suggesting that mammary-specific elements reside in 2.6 kb of the promoter upstream region. However, correct developmental and hormonal regulation of the chimeric genes was not observed (5). To test whether sequences downstream from the promoter are required for correct regulation of the WAP gene, we have introduced into mice a 7.2-kb fragment of DNA which encompasses the entire transcribed region of the mouse WAP gene, 2.6 kb of 5'- and 1.6 kb of 3'-flanking DNA. Analysis of WAP transgene expression in six independent lines of mice allowed us to study the contribution of regulatory elements within the 7.2 kb to the developmental and hormonal regulation of the mouse WAP gene. In addition, the introduction of three different WAP gene alleles into a single integration site allowed us to evaluate the influence of surrounding chromatin on the level of expression and developmental regulation of individual transgenes within one locus.

MATERIALS AND METHODS

Recombinant Plasmids—The plasmid pBS WAP, containing a 7.2-kb EcoRI fragment with the mouse WAP gene (14), was linearized...
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with either KpnI (exon 1), SalI (exon 3), or BamHI (exon 4), blunted, and ligated with either HindIII, SalI, or NdeI linkers. Three WAP linker alleles were generated: the KH allele contained one HindIII linker in the KpnI restriction site; the SSM allele contained one SalI linker in the SalI restriction site; and the BN allele had five NdeI linkers inserted into the BamHI restriction site (Fig. 1).

Templates used to generate synthetic RNA transcripts containing both the linker-allele and the corresponding wild-type sequences were constructed by subcloning an exon containing the linker and the equivalent wild-type sequence oligonucleotide into the plasmid pBS. Plasmid pHpK contains the first exon of the KH WAP allele and a copy of the corresponding wild-type sequence; plasmid pSIM contains wild-type and allelic sequences spanning the SalI site; and plasmid pBmD contains wild-type sequence oligonucleotides specific for either the allelic or corresponding wild-type sequence. The hybridizations were carried out as described (16). Polyadenylated RNA was prepared by a batch method (17). Expression of the WAP gene in organ culture was 100 ng/ml of insulin and hydrocortisone and 1 pg/ml of prolactin.

RESULTS

Generation of Transgenic Mice—WAP alleles were separated from vector sequences by digestion with EcoRI, followed by electrophoresis in an agarose gel (FMC). DNA fragments were then isolated by electroelution and purification on a ion-exchange column (NACS prep; Bethesda Research Laboratories). The fragments were microinjected into the pronuclei of zygotes obtained from C57BL6/SJL F1 and pBmD. The dots were excised, and the bound radioactivity was quantitated by scintillation counting. Results were normalized against a standard sample of RNA obtained from a transgenic lactating mouse was included on blots to demonstrate the specificity of the oligonucleotides. Based on the radioactivity associated with a band on the filter was quantitated by scintillation counting. In order to measure the relative levels of transgene expression, as well as any serial dilution of an equimolar mixture of three synthetic transcripts generated in vitro from plasmids pHpK, pSIM, and pBmD. In addition a standard sample of RNA obtained from a transgenic lactating mouse was included on blots to demonstrate the specificity of the oligonucleotides. Based on the results of Southern or PCR analysis, transgenic offspring were produced by breeding the founders with C57BL6/SJL F1 female mice. Two classes of mice were produced, KH mice were generated by injection of the KH allele alone, and KSB mice were produced using an approximately equimolar mixture of the three alleles.

DNA Analysis—Transgenic founder mice were identified by Southern blot analysis of tail DNA. DNA was prepared from tail tissue by digestion with proteinase K and precipitation with ethanol. The phenol/chloroform extractions were eliminated without adversely affecting the results of Southern or PCR analysis. Transgenic offspring were produced by breeding the founders with C57BL6/SJL F1 mice. Once a stable line was established, offspring were screened by PCR. Mice carrying the KH allele were identified by amplification of the first WAP exon and testing the product for the presence of a HindIII restriction site. Amplification of the fourth WAP exon, which contains a HindIII linker in the KpnI site and the SalI site; and the BN allele contained five NdeI linkers in the BamHI site (Fig. 1). The 0.65-kb WAP mRNA transcribed from the different WAP gene alleles was detected by hybridization with antisense oligonucleotides specific for the leader peptide. The mouse keratin 18 probe was generated by random priming of a cDNA insert prepared from plasmid pUC97B (18), that had been kindly provided by Dr. Robert Oshima.

Generation of Mice Carrying Mouse WAP Transgenes—To distinguish between the endogenous and transgenic mouse WAP mRNAs, we tagged the transgenes by inserting linkers into either exons 1, 3, or 4 of the mouse WAP gene. Three linker alleles were produced: the KH allele contained a HindIII linker in the KpnI site; the SSM allele had a SalI linker at the SalI site; and the BN allele carried five NdeI linkers at the BamHI site (Fig. 1). The 0.65-kb WAP mRNA transcribed from the different WAP gene alleles was detected by hybridization with antisense oligonucleotides specific for the linker insertions.

Two classes of transgenic mice were produced; KH mice, which carried KH alleles, and KSB mice, which contained all three alleles. Transgenic mice containing the three alleles were generated to evaluate whether insertion of any of the linker molecules would interfere with gene expression. From five KH and fifteen KSB founder mice, thirteen lines of mice transmitted the transgenes to their offspring. Of these founder mice, three KH (3350A, 3350B, 3350C) and four KSB lines

FIG. 1. Structure of the WAP gene alleles. A 7.2-kb genomic fragment spanning the WAP gene was modified by the insertion of oligonucleotide linkers into either the KpnI (K), SalI (S), or BamHI (B) restriction sites, producing three different alleles, KH, SSM, and BN, respectively. The open and closed boxes represent untranslated and translated regions of the four WAP gene exons, respectively.
(3336, 3441, 3628, 3631) expressed the WAP transgenes during lactation at levels between 3 and 54% of the endogenous WAP gene. Expression was confined to the mammary gland (data not shown). A comparison of WAP transgene copy number and expression between six of these lines indicated that the transgene was expressed in a position dependent and copy number independent manner (Table I). Apparently the 7.2-kb WAP transgene does not represent a complete regulatory unit. However, expression levels of this construct were at least an order of magnitude higher than of hybrid genes containing just the WAP gene promoter.

Since the three WAP gene alleles cointegrated in KSB mice in a tandem head-to-tail arrangement (data not shown), we were able to investigate whether expression of the transgenes, which was sensitive to the surrounding chromatin (Table I), was also subject to position effects within the transgene locus. The copy number for each transgenic allele within the loci of the KSB lines was determined by quantitative PCR and was correlated with the steady state levels of the different RNA transcripts (Table II). In the three lines tested, expression of the WAP SSM and BN transgene alleles was proportional to their copy number within the transgene array, suggesting that alleles within a locus are equally active. However, the two copies of KH alleles of line 3336 were only expressed at 50% of the other alleles, and the single copy of KH allele in line 3441, situated at the border of the transgene cluster as judged by Southern analysis (data not shown), was also expressed at significantly lower levels than the SSM and BN alleles. Taken together the results suggest that most of the WAP transgenes within a transgene locus were expressed and that the level of expression among them was similar. Nevertheless, consistently lower expression of KH transcripts in different lines may indicate that the HindIII linker did interfere quantitatively with expression of the transgene.

Regulation of WAP Transgene Alleles during Development—RNA prepared from the mammary glands of female transgenic mice at various stages of development, from virgin through pregnancy and lactation, was analyzed on replicate Northern blots for either the endogenous WAP mRNA or transcripts from the different WAP transgene alleles. The endogenous gene was induced between 13 and 17 days of pregnancy and expression peaked around mid-lactation (Figs. 2 and 3). However, the expression of WAP transgenes differed qualitatively and quantitatively from the endogenous gene (Fig. 2). The developmental patterns obtained with lines 3350B and 3336 illustrate two general aspects of WAP transgene expression. First, although the absolute levels of transgene expression differed between lines of mice, the patterns of induction during pregnancy were similar. Transgenic WAP mRNA was detected at day 13 of pregnancy, preceding induction of the

![Figure 2](image-url)
enogenous gene by at least 2 days. Furthermore, whereas the enogenous gene is induced about 1000-fold between day 13 of pregnancy and parturition, expression of the transgenes during this period in all lines, except 3350 C, increased less than 10-fold. Second, the activities of the WAP transgenes within one line was made possible by correcting for the different hybridization efficiencies of oligonucleotides using synthetic RNA standards (see “Materials and Methods”).

**TABLE III**

<table>
<thead>
<tr>
<th>Line</th>
<th>Allele</th>
<th>Expression of allele as percentage of endogenous WAP</th>
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<td>P13</td>
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<tr>
<td>3350 A</td>
<td>KH</td>
<td>850</td>
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<tr>
<td>3350 B</td>
<td>KH</td>
<td>1080</td>
</tr>
<tr>
<td>3336</td>
<td>SSM</td>
<td>180</td>
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<tr>
<td>3441</td>
<td>BN</td>
<td>24</td>
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<tr>
<td>3350 C</td>
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* ND, not determined.

endogenous by at least 2 days. Furthermore, whereas the endogenous gene is induced about 1000-fold between day 13 of pregnancy and parturition, expression of the transgenes during this period in all lines, except 3350 C, increased less than 10-fold. Second, the activities of the WAP transgenes during lactation differed dramatically between lines of mice. Expression either continued to increase approximately to the same extent as the endogenous gene as seen in lines 3350 A and B, or expression declined as in lines 3336, 3441 and 3350 C (Figs. 2 and 3). Presenting the concentration of transgenic WAP mRNA as a percentage of the endogenous mRNA further emphasizes the premature expression of transgenes, the similarities between the patterns of transgene expression during pregnancy, and differences during lactation (Table III).

The aberrant developmental expression of the WAP transgene alleles in different lines, especially during lactation, does not appear to be caused by the linker insertions or by differential expression of individual WAP genes within transgene loci. The three WAP gene alleles in the KSB lines 3336, 3441, and 3628 were integrated in a single locus, and expression during mammary development was coordinately regulated as representatively shown for line 3336 (Fig. 2).

**In Vitro Hormonal Regulation of the WAP Transgenes in Mammary Tissue from Pregnant Mice**—To correlate the induction of the transgenes during development to hormonal regulation, we analyzed the expression of the WAP transgenes in an organ culture system. The WAP transgenes in lines 3350B and 3336 were already active in mammary tissue from mice which were 13–15 days pregnant (Fig. 4). In the presence of insulin, hydrocortisone, and prolactin, transgene expression in tissue from mice of lines 3350B and 3336 increased 4- and 2-fold, respectively (Fig. 4). Under the same conditions the endogenous WAP gene was induced over 30-fold. Expression of the WAP transgenes in tissue from mid-pregnant animals in the presence of insulin alone was below the level of detection, but was maintained in the presence of insulin and hydrocortisone and to a lesser extent with insulin and prolactin (Fig. 4). The levels of transgene mRNA detected in explants incubated with insulin and hydrocortisone were not due to retention of pre-existing mRNA but arose from continuous transcription of the transgenes, since comparable levels endogenous WAP mRNA were not maintained under these conditions in organ explants from late pregnant and lactating animals (Fig. 5 and data not shown).

Rehybridizations of RNA from explant cultures with a probe specific for the mouse β-casein mRNA demonstrated that, in common with the WAP transgenes, significant levels of β-casein mRNA were present at day 13 of pregnancy; induction in the presence of insulin, hydrocortisone, and prolactin (IFP) was only about 4-fold (Fig. 4). However, whereas expression of the WAP transgene appears to be more dependent on hydrocortisone than prolactin, the reverse is true for the β-casein gene (Fig. 4). Furthermore, the similarity between the β-casein blots from different explant experiments indicated that the response of the transgenes in different lines of mice can be compared directly.

**In Vitro Hormonal Regulation of the WAP Transgenes in Mammary Tissue from Lactating Mice**—The decline in expression of WAP transgenes during lactation in lines 3336,
Transgenic mice provide a unique tool for studying the developmental regulation of genes (19-21), especially those such as the milk protein genes, which are only appreciably expressed in terminally differentiated cells (5, 22-24). We introduced a virtually unaltered 7.2-kb fragment, including the entire transcribed region of the mouse WAP gene, into mice and studied its developmental and hormonal regulation. The level of expression of the WAP transgene was at least an order of magnitude higher than several hybrid genes containing the WAP promoter (4, 5, 13, 25, 26). However, expression during mammary development and upon hormonal stimulation in vitro differed from the endogenous WAP gene. By comparing the developmental patterns of expression, and hormonal regulation of the WAP transgenes in six lines of mice, novel aspects of WAP gene regulation became apparent.

In contrast to the variation in overall patterns of transgene expression during mammary development, and especially during lactation (Fig. 3), the activity of WAP transgenes during mid-pregnancy in different lines of mice was similar. In all lines examined, expression of the WAP transgenes was detected at day 13 of pregnancy, thereby preceding the induction of the endogenous gene. Premature activation during pregnancy has also been observed with a hybrid gene containing the mouse WAP gene promoter (5) and a rat WAP transgene (27). Furthermore, between day 13 of pregnancy and parturition, a period in which the endogenous WAP gene was induced almost 1000-fold, expression of WAP transgenes in all lines except one increased less than 10-fold.

Results from organ culture experiments with mammary tissue from pregnant mice provided an insight into the basis of the temporal deregulation of the WAP transgene. The premature expression of WAP transgenes during pregnancy was presumably related to the high basal activity of the transgene; the activity was maintained in explant cultures with hydrocortisone and insulin, both of which are present throughout pregnancy. These observations suggest that the

**Fig. 4.** Hormonal regulation of WAP transgenes in mammary tissue from mid-pregnant mice. Mammary explants were prepared from 13 to 15-day pregnant mice and RNA was extracted at the time of preparation (To) or after 48 h in culture in the presence of insulin (I), insulin and hydrocortisone (IF), insulin and prolactin (IP), or insulin, hydrocortisone, and prolactin (IFP). Northern blots carrying 5 μg of total RNA/lane were hybridized with oligonucleotides specific for either WAP alleles (upper panel) or mouse β-casein (lower panel).

3441, and 3350 C raised the question whether expression of the transgenes was generally repressed or had lost its response to a particular hormone. Tissue explants prepared from a fully lactating mouse from line 3336 were cultured under the conditions employed in the induction experiments shown in Fig. 4. Although the level of endogenous WAP RNA decreased at least 10-fold under any of the hormonal conditions, levels of the transgenic mRNA either increased marginally in the presence of insulin, hydrocortisone, and prolactin (IFP) or remained the same with insulin and either hydrocortisone or prolactin (Fig. 5). Expression of keratin 18, a gene expressed in simple epithelial cells (18), increased during organ culture and was unaffected by hydrocortisone or prolactin. The overall response of the transgenes to hormones in tissue from lactating mice from line 3336 was similar to that obtained with tissue from pregnant mice. These results support the notion that the decrease in expression of the WAP transgenes during lactation in line 3336, and presumably also in lines 3441 and 3350 C, was due to a lack of sensitivity to the regulatory factors responsible for WAP gene induction during lactation. The transgenic WAP mRNA present during lactation in these lines probably reflects a basal level of transcription.

A similar result was obtained in vivo with mammary tissue from mice of line 3336 where, after 3 days of weaning, levels of the endogenous WAP mRNA decreased about 100-fold and expression of transgenic WAP mRNA remained constant (data not shown).

**Fig. 5.** Hormone-dependent expression of WAP transgenes in mammary tissue from lactating mice of line 3336. Mammary explants were prepared from a 10-day lactating 3336 mouse, and RNA was extracted at the time of preparation (To) or after 48 h in culture in different combinations of insulin (I), hydrocortisone (F), and prolactin (P). Northern blots carrying 5 μg of total RNA/lane were hybridized with oligonucleotides specific for either WAP alleles or a DNA probe generated from a mouse keratin 18 cDNA (12).
transgene lacks a repressor element, or alternatively, the transgene array or surrounding chromatin results in the functional loss of a repressor. Consistent early expression and variation in both patterns of induction during pregnancy and response to hormones in vitro suggest that both situations may operate. Whereas the 30-fold induction of the endogenous WAP gene in vitro required the synergistic action of insulin, hydrocortisone, and prolactin, expression of the transgenes was only marginally induced by prolactin. The small in vitro induction of the WAP transgenes by prolactin correlates with the modest increase in expression prior to parturition and is presumably a consequence of the prematurely elevated expression levels in mid-pregnancy. In a manner similar to the response of a WAP-tPA transgene (5), prolactin was not necessary to maintain the level of transgenic WAP RNA in organ culture, but was required for continued expression of the endogenous WAP gene. Taken together the results are consistent with prolactin acting on the endogenous WAP gene through the release of repression, thereby facilitating access of the gene to regulatory factors dependent on insulin and hydrocortisone. In support of this model, results obtained with a cell culture system suggest that the mouse β-casein promoter is induced by hormones through the release of transcriptional repression.4

In contrast to the situation in pregnancy, expression of the WAP transgenes in individual lines of mice differed dramatically during lactation. In two lines expression of the WAP transgene approximately followed the pattern of the endogenous WAP gene. However, in three other lines of mice, in which the transgenes were induced during pregnancy and responded to hormones in organ culture, expression decreased in response to lactation. To obtain the same effect in three independent lines of mice demonstrates that loss of induction was not peculiar to one site of integration, but that the transgene lacks a regulatory element necessary to ensure correct expression during lactation. Günzburg and co-workers (28) have also reported the down-regulation of a WAP-hGH transgene in transgenic mice. Although a decrease in the expression of a 4.3-kb rat WAP transgene during lactation was not reported (27), this phenomenon may neither have been detected, because developmental studies were performed on only two lines of mice and at a single time point during lactation. Alternatively, the rat and mouse WAP genes may have a different arrangement of regulatory elements.

Our results indicate that high level WAP gene expression during lactation is mediated by a combination of elements or mechanisms distinct from those operating during pregnancy. This is supported by the inability to maintain high levels of endogenous WAP RNA in tissue from lactating mice, under conditions sufficient to induce expression in tissue from pregnant mice. Expression of keratin 18 mRNA in culture (Fig. 5) demonstrated that the decrease in endogenous WAP mRNA in vitro was not due to a general decrease in gene expression. Appropriate induction of WAP transgenes in two lines of mice (3350 A, 3350 B) during lactation indicates that chromatin at the sites of integration may have provided a positive influence which enabled the transgene to be appropriately induced during lactation. A similar facilitating effect of flanking chromatin has been described in a deletion anal-

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