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Genetics

Matrix-attachment regions can impart position-independent regulation of a tissue-specific gene in transgenic mice

(whey acidic protein gene/*A*-elements/chromatin/genetic domains/mammary gland)

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ABSTRACT **Matrix-attachment regions (MARs) may function as domain boundaries and partition chromosomes into independently regulated units. We have tested whether MAR sequences from the chicken lysozyme locus, the so-called *A*-elements, can confer position-independent regulation to a whey acidic protein (WAP) transgene in mammary tissue of mice. In the absence of MARs, expression of WAP transgenes was observed in 50% of the lines, and regulation during pregnancy, during lactation, and upon hormonal induction did not mimic that of the endogenous WAP gene and varied with the integration site. In contrast, all 11 lines in which WAP transgenes were juxtaposed to MAR elements showed expression. Accurate position-independent hormonal and developmental regulation was seen in four out of the five lines analyzed. These results indicate that MARs can establish independent genetic domains in transgenic mice.**

Expression of transgenes is, in general, unpredictable and varies with the chromosomal site of integration. This position effect may reflect the organization of chromatin into topologically constrained loops that serve to define functional genetic domains (see refs. 1–3). Such loops are thought to be established by matrix- or scaffold-attachment regions (MARs or SARs). MARs have been identified at the boundaries of functional transcription units from several species (1, 4–6), and they have been shown to buffer effects of flanking chromatin in stably transfected cell lines (7, 8). However, whether MARs can buffer eukaryotic genes in transgenic animals is unknown.

The whey acidic protein (WAP) gene is a good candidate for testing the possible function of MARs because its developmental and hormonal regulation in transgenic animals is highly position dependent. The WAP gene encodes a major milk protein, and its expression during pregnancy, lactation, and involution is regulated by multiple developmental and hormonal signals (9, 10). Expression of WAP transgenes has resulted in mammary-specific transcription, but levels were variable, and the transgenes were activated precociously during pregnancy (9, 11–14) and, in some cases, turned off during lactation. In addition, transgene expression was constitutive and hormone-independent (9, 10). Here, we report on the ability of a chicken lysozyme MAR to buffer a mouse WAP transgene from position effects in transgenic mice.

EXPERIMENTAL PROCEDURES

Generation of Transgenes and Mice. The 7-kb mouse WAP transgene was identical to that used earlier (15), except that it contained the WAP protein-coding region from the YBR strain (16). The *Hin*dIII linker in the *Kpn* I site in the 5′-untranslated region enabled us to distinguish between endogenous and transgenic WAP RNA (9) and to distinguish the endogenous from the transgenic WAP genes. The chicken lysozyme 5′ MARs were isolated as a 3-kb *Bam*HI–*Xba* I fragment (8).

Seven hundred and seventy-two zygotes were injected with the WAP transgene and transferred into 36 foster mothers, of whom 18 gave birth. The transgene was detected in 19 (17%) of the 111 pups born. Nine hundred and eighteen zygotes, coinjected with the MAR and WAP gene fragments (in a 2:1 molar ratio), were transferred into 41 foster mothers. Twenty-two of those recipients gave birth to 152 pups. The WAP transgene was detected in 27 (15%) of the pups born.

Transgenic mice were identified by using PCR analysis. The first exon of the endogenous and transgenic WAP genes was amplified (9) and restricted with either *Hin*dIII or *Kpn* I. Transgenic mice, identified by successful *Hin*dIII digestion of the PCR fragment, were further analyzed by Southern blot analysis. The WAP 5′-probe was a 1.1-kb *Sst* I fragment spanning promoter sequences from −1450 to −350. The MAR 5′-probe was a subcloned 0.5-kb *Bam*HI–*Hin*dIII fragment, and the 3′-probe was a subcloned 1.3-kb *Hin*dIII–*Xba* I fragment (7, 8).

Transgene copy number was determined by using PCR. The first exon of the endogenous and transgenic WAP genes was amplified from 5-fold serial dilutions of template DNA for 25 cycles in 50-μl reaction volumes. A single ^{32}P-end labeled primer at 3.6×10^{-2} μmol was added with 3.6 μmol of each unlabeled primer. Equivalent samples of each dilution were digested with *Kpn* I and *Hin*dIII, which restricts the endogenous and transgenic alleles, respectively. The digests were electrophoresed in 2% agarose gels, and counted with an Ambis β-counter.

Analysis of Transgene Expression. Expression of WAP transgenes was initially examined within 48 hr after parturition. Transgene expression during pregnancy, lactation, and involution was analyzed in five WAP/MAR lines. WAP transgene expression during pregnancy and lactation in mice carrying only WAP transgenes was established recently in six lines (9, 13), and involution was examined in two of these lines. Total RNA was isolated from mammary tissue (17) and 20 μg of total RNA was separated in formaldehyde gels, blotted onto a nylon membrane, and hybridized with ^{32}P-labeled oligonucleotides complementary to the transgene or endogenous WAP RNA or β-casein mRNA (9). Mammary organ explants were prepared as described (10). Concentrations of hormones in organ culture were 100 ng/ml for insulin and hydrocortisone and 1 μg/ml for prolactin.

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Abbreviations: MAR, matrix-attachment region; WAP, whey acidic protein.

Table 1. Expression and regulation of WAP transgene compared with endogenous WAP

Line	WAP transgene copy no.	Expression, % Total*	Per copy†	Regulation Developmental‡	Hormonal§
Group I					
7442	3	25	8	Yes	NT
7449	50	56	1	Yes	Yes
7499	9	21	2	NT	NT
7603	3	27	9	NT	NT
7605	8	5	<1	NT	NT
7606	2	17	9	Yes	Yes
7612	5	1	<1	No	No
7676	2	10	5	NT	NT
7682	2	31	16	Yes	Yes
7719	3	35	14	Yes	Yes
7767	9	27	3	NT	NT
Group II					
7519	NT	11	NT	NT	NT
7521	3	24	8	NT	NT
7541	9	<1	<1	NT	NT
7542	30	25	<1	NT	NT
7695	10	1	<1	NT	NT
7707	19	125	7	No	NT
Group III					
3350A	11	45	4	No	No
3350B	15	54	4	No	No
3350C	7	3	<1	No	NT
3336	12	46	4	No	No
3628	20	34	2	No	No
3441	14	18	1	No	NT

Group I are transgenic mice carrying WAP transgenes and *A*-elements; expression data is from 11 of 11 lines analyzed. Group II are transgenic mice carrying only WAP transgenes (this study); expression data are from the 6 of 12 lines analyzed that expressed transgene at a level of at least 1% of endogenous WAP. Group III are transgenic mice carrying only WAP transgenes; expression data are from 7 of 13 lines analyzed that expressed transgene at a level of at least 1% of endogenous WAP (data from Burdon *et al.*, ref. 9). NT, not tested.

*Percent WAP transgene expression compared with total endogenous WAP RNA.

†Percent per copy of WAP transgene compared with total endogenous WAP RNA.

‡Mimics endogenous WAP RNA expression during pregnancy, lactation, and involution.

§Mimics endogenous WAP RNA expression in the presence of insulin; insulin and hydrocortisone; insulin and prolactin; and insulin, hydrocortisone, and prolactin.

RESULTS

Transgenic Mice with Mouse WAP Transgenes and MAR Sequences. To evaluate the effects of MARs on the regulation of WAP transgenes in the context of different chromosomal loci, we attempted to generate recombinant plasmids containing the mouse WAP gene flanked by MARs from the chicken lysozyme locus. However, when recombinant plasmids isolated from bacteria showed extensive rearrangements (data not shown), we elected to coinject the two fragments, the WAP gene and MARs, into mouse zygotes. From the 14 founders analyzed, 11 contained several copies of MARs in the transgenic WAP locus; those lines will be referred to as WAP/MAR lines. The WAP transgene was also injected independently, and nine lines were analyzed. The copy number of WAP transgenes in both the WAP and WAP/MAR lines was between 2 and 9, except for the WAP/MAR line 7449, which contained ≈50 copies of the WAP gene (Table 1). Colinearity of WAP and MAR sequences in transgenic loci was verified by Southern blot and PCR analyses. A Southern analysis from lines 7682, 7719, and 7612 is shown in Fig. 1. Genomic DNA restricted with

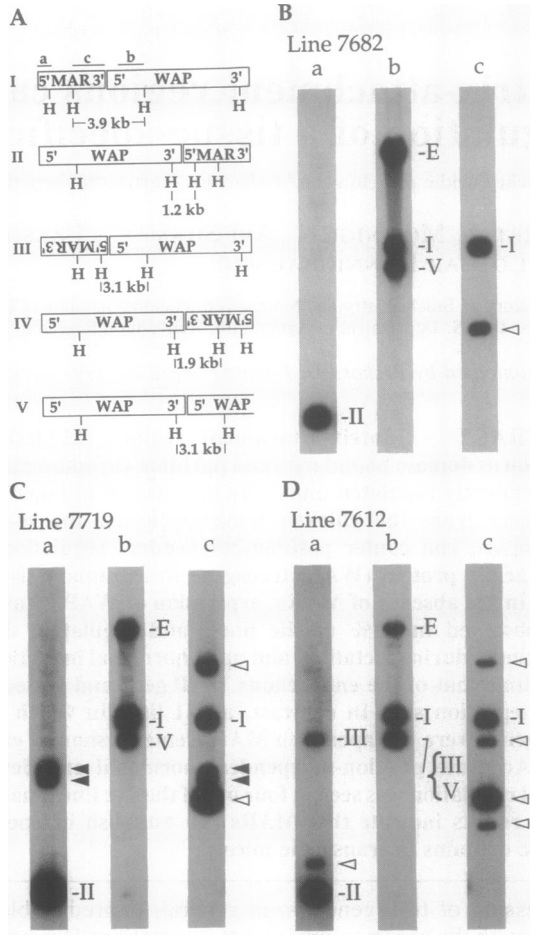

FIG. 1. Possible arrangements of WAP and MAR sequences upon cointegration into transgenic loci (*A*) and Southern blot analyses of genomic DNA from lines 7682 (*B*), 7719 (*C*), and 7612 (*D*). *Hin*dIII restriction sites are indicated as H, and the sizes of restriction fragments detected with different probes are indicated. Open triangles probably represent MAR fragments at the site of integration and rearranged fragments. The fragments indicated by open triangles, which hybridize with the MAR 3′-probe also hybridized with the subcloned internal *Hin*dIII fragment from the MARs (data not shown). Solid triangles in line 7612 represent MAR elements in head-to-tail orientation. The filters were first hybridized with the MAR 5′-probe (probe a), stripped and rehybridized with the WAP probe (probe b), and then stripped again and rehybridized with the MAR 3′-probe (probe c). The faint 1.2-kb band seen in *b*, is from incomplete stripping after hybridization with MAR 5′-probe (*a*).

*Hin*dIII and probed with a WAP 5′-probe resulted in a 20-kb band representing the endogenous WAP gene. The 3.9-kb band seen in all three lines indicated a head-to-tail arrangement of MAR and WAP sequences (Fig. 1*A*, configuration I, and lanes b of Fig. 1*B*, *C*, and *D*). This interpretation was confirmed by reprobing the filter with a 3′-MAR probe (lanes c of Fig. 1*B*, *C*, and *D*). The 3.1-kb band obtained with the WAP probe represented head-to-tail arrangements of WAP transgenes (Fig. 1*A*, configuration V, lanes b of Fig. 1*B*, *C*, and *D*). In line 7612 a head-to-head ligation of MAR and WAP sequences resulted in a band of identical size to a WAP head-to-tail arrangement (Fig. 1*A*, configuration III, and lane a of Fig. 1*D*). The 1.2-kb band hybridizing with the 5′-MAR probe represented MAR elements ligated to the 3′ end of WAP genes (Fig. 1*A*, configuration II, and lanes a of Fig. 1*B*, *C*, and *D*). Given the intensity of the 3.9-kb band (MAR/WAP head-to-tail) relative to the 1.2-kb band (WAP/MAR head-to-tail), it is likely that WAP and MAR sequences integrated in a tandem array.

With a WAP 5′-probe all fragments could be accounted for by WAP head-to-tail and different WAP–MAR arrangements (Fig. 1), suggesting that WAP promoter sequences were not juxtaposed to flanking host chromatin. Because the 3′ end of the WAP gene consists of middle repetitive elements, it was not possible to test whether 3′-flanking sequences were located at the 3′ end of the WAP gene. However, with MAR probes in most, if not all, cases MARs are clearly located at the 3′ end of the WAP gene. The presence of fragments that hybridize with 5′ and 3′ MAR sequences, have the intensity of single-copy genes and cannot be accounted for by any arrangement of WAP and MAR sequences, indicate integration sites into the transgene locus. Some of these fragments also hybridized with internal MAR sequences, suggesting that rearrangements had occurred within MAR sequences. Some fragments hybridizing with the 3′-MAR probe also hybridized with the subcloned internal *Hin*dIII fragment from the MAR element (marked by open triangles in Fig. 1*B*, *C*, and *D*), suggesting that rearrangements within MAR sequences had occurred. No rearrangements were found in the WAP transgene portion. Line 7682, in which the WAP transgene was accurately regulated, showed little MAR rearrangement (Fig. 1*B*), but extensive MAR rearrangements were found in line 7612 (Fig. 1*D*), which showed deregulated transgene expression. MAR elements arranged in a head-to-tail fashion were only found in line 7719 (solid triangles in Fig. 1*C*). Although most WAP transgenes were located next to MARs, the presence of multiple WAP gene copies prohibited exact deciphering of the arrangement within the transgene locus. Line 7682 contained only two head-to-tail-arranged WAP transgene copies, which were flanked on either side by MARs.

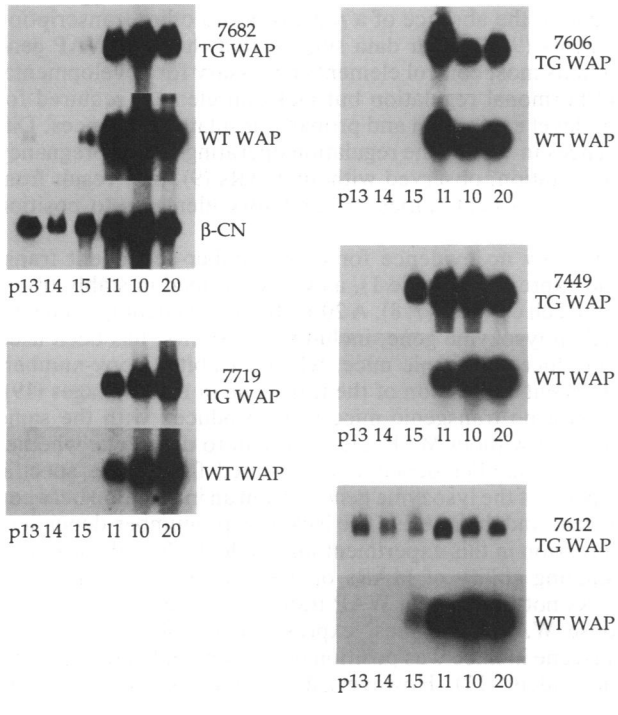

FIG. 2. Accumulation of endogenous and transgenic WAP RNA during pregnancy and lactation in lines carrying WAP/MAR transgenes. RNA from the WAP/MAR lines was prepared from mammary tissue at the appropriate developmental stage, and the respective RNA blots were successively hybridized with oligonucleotide probes detecting transgene WAP, endogenous WAP, and β-casein transcripts. Blots were stripped between hybridizations. Each line is indicated by a composite figure. Timepoints p13, 14, and 15 refer to day of pregnancy at which tissue was taken, and l1, 10, and 20 refer to day of lactation. WT WAP, endogenous WAP RNA; TG WAP, transgenic WAP RNA; β-CN, β-casein RNA.

Frequency of WAP Gene Expression. In a previous study 6 of 11 mouse lines carrying mouse WAP transgenes expressed the transgene at >1% of the endogenous level (9). In this study, WAP transgene RNA was found in 6 out of 12 lines that carry only the WAP transgene, whereas all 11 of the WAP/MAR lines, with intact copies, expressed the transgene. However, steady-state levels of transgene WAP RNA in the WAP/MAR mice did not appear higher than in the lines that only carried WAP transgenes, and no copy number-dependent expression was seen (Table 1; only lines that express the transgene at >1% of the endogenous level are shown). The presence of MARs did not alter the mammary specificity of WAP gene expression (data not shown).

WAP Gene Expression During Mammary Development. Previous studies have shown that WAP transgenes were expressed prematurely during pregnancy (9, 11–13). Although the endogenous WAP gene was induced >100-fold between day 13 of pregnancy and day 2 of lactation, activation of transgenes was <10-fold (9). In contrast, in four out of the five WAP/MAR lines tested, the pattern of transgene expression mimicked that of the endogenous gene—i.e., little or no WAP transgene expression was detected at day 13 of pregnancy (Fig. 2). In three of the four WAP/MAR lines transgene expression remained low until day 15 of pregnancy, followed by a sharp increase by day 1 of lactation. The transgenic, but not endogenous, WAP RNA was detected at day 15 of pregnancy in line 7449.

In three out of five lines carrying only mouse WAP transgenes steady-state levels of transgenic RNA dropped sharply during lactation while the endogenous counterpart was fully induced (ref. 9 and Table 1). In four of the five

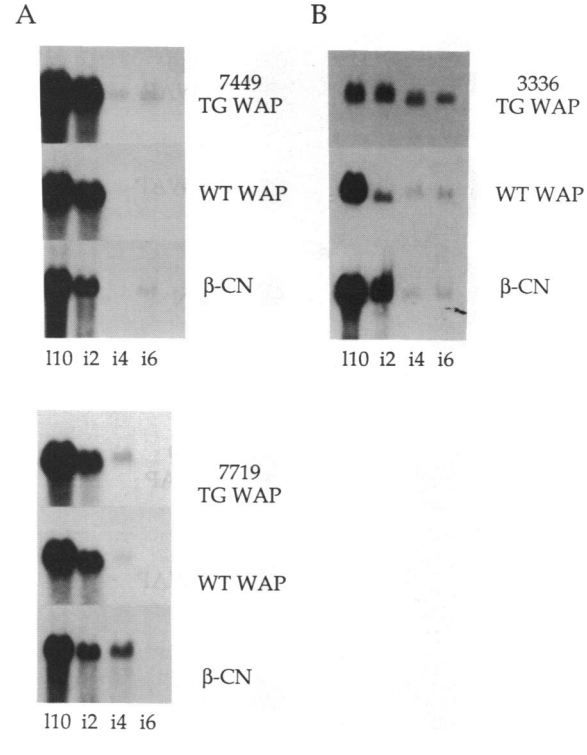

FIG. 3. Accumulation of endogenous and transgene WAP RNA during involution in lines carrying WAP/MAR (*A*) and WAP (*B*) transgenes. Pups were separated from their mothers at day 10 of lactation, and RNA was prepared from mammary tissue at weaning and 2, 4, and 6 days thereafter. RNA blots were successively hybridized as illustrated. Results from WAP/MAR lines 7606 and 7682 (data not shown) were indistinguishable from the data from WAP/MAR lines 7449 and 7719. Line 3336 appears to be a milchlos line (9). l10, Day 10 of lactation; i2, i4, and i6, day 2, 4, and 6 after separation from pups. See legend for Fig. 2.

WAP/MAR lines analyzed expression of the transgenes during lactation paralleled that of the endogenous counterpart (Fig. 2). Deregulated expression was seen in line 7612, which also showed rearrangements within the transgene locus. Steady-state levels of transgene RNA in this line were low, and little induction was seen between day 13 of pregnancy and day 20 of lactation (Fig. 2).

After weaning mice at day 10 of lactation, the mammary gland involutes rapidly, and steady-state levels of endogenous WAP RNA drop below 1% within 4 days (Fig. 3*A*). In contrast, transgene expression in WAP lines 3336 and 3350 (Fig. 3*B* and Table 1), which did not harbor MARs, was maintained for 6 days after weaning. In addition to deregulated transgene expression, line 3336 showed evidence of endogenous WAP and β-casein gene deregulation (Fig. 3*B*). Deregulated WAP gene expression accompanied by lactation failure has also been seen in transgenic swine (18). In four WAP/MAR lines the WAP transgenes, like the endogenous WAP gene, were rapidly downregulated during involution (Fig. 3*A* and Table 1). Steady-state levels of β-casein mRNA decreased with a similar kinetics as WAP.

***In Vitro* Induction of WAP Transgenes in Mammary Tissue from Pregnant Mice.** Expression of the endogenous WAP gene in mammary tissue from mice at mid-pregnancy can be induced >50-fold during *in vitro* culture in the presence of insulin, hydrocortisone, and prolactin (refs. 9 and 10; Fig. 4). Although synergistic activity of insulin, hydrocortisone, and prolactin was necessary to activate the endogenous gene, transcription of the transgenes, in lines without MARs, was observed in the presence of insulin and prolactin or of insulin and hydrocortisone (9). In contrast, strong induction of WAP transgenes in mammary cultures from mice at mid-pregnancy

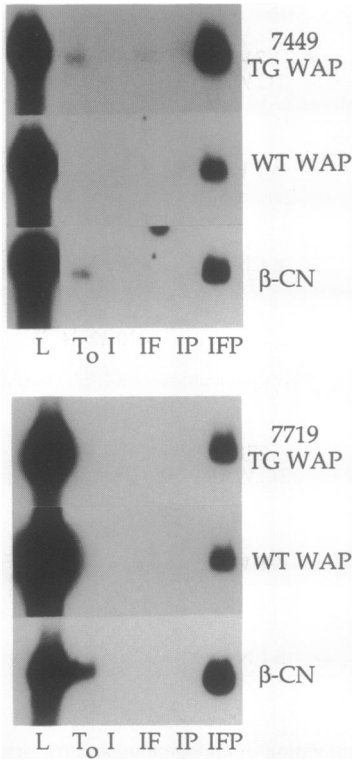

FIG. 4. *In vitro* induction of endogenous and transgene WAP mRNA in mammary tissue from pregnant mice from lines carrying WAP/MAR transgenes. Mammary explants were prepared from mice at day 14 of pregnancy, and RNA was extracted at the time of necropsy (T_0) or after 48 hr in culture in the presence of insulin (I), insulin and hydrocortisone (IF), insulin and prolactin (IP), or insulin, hydrocortisone, and prolactin (IFP). RNA blots were successively hybridized as described for Fig. 2. L, lactation.

from four WAP/MAR lines analyzed was obtained in the presence of insulin, hydrocortisone, and prolactin (Fig. 4). Although induction of the transgene in lines 7719 (Fig. 4), 7606 and 7682 (data not shown) paralleled that of the endogenous WAP gene, the transgene in line 7449 was activated differently (Fig. 4). WAP transgene expression in line 7449 was seen as early as day 15 of pregnancy, suggesting that the transgene at day 14 of pregnancy may have been in a preactivated state and the threshold for activation by the insulin–hydrocortisone–prolactin signaling pathways was lower than for the endogenous gene.

DISCUSSION

It is possible that transgenes are susceptible to chromosomal position effects because they fail to establish domains of independent gene activity. Here we have shown that MARs from the chicken lysozyme locus can insulate a heterologous gene from such position effects in transgenic mice. Expression of the transgene was found in all mouse lines in which intact MARs and WAP transgenes had cointegrated but was detected in only about half of the lines without MARs. Similarly, the chicken lysozyme MAR conferred elevated and less position-dependent expression on homologous and heterologous promoters in stably transfected tissue culture cells (7, 8). No increase in expression levels was seen in this study, supporting earlier observations that MARs do not function as enhancers (8).

The presence of MARs restored the developmental regulation of WAP transgenes, and accurate regulation during pregnancy coincided with *in vitro* hormonal inducibility. The loss of stringency in WAP transgene expression during pregnancy and upon hormonal stimulation has been suggested to be due to the absence of a repressor and other transcription elements (9–12). Our data suggest that the 7-kb WAP gene contains most control elements necessary for developmental and hormonal regulation but lacks an element required for high-level expression and probably insulator sequences. Differences in WAP gene regulation operating during pregnancy and lactation, observed without MARs (9), may result from different susceptibilities of regulatory elements to position effects.

We saw no evidence for copy-number-dependent transgene expression (Table 1), as was described in stably transfected cell cultures (7, 8). A 20-kb fragment encompassing the chicken lysozyme gene, including its MARs, has been used to produce transgenic mice, which exhibited copy-number-dependent expression of the transgene in macrophages (19). Because no transgenic mice were produced with the same construct without MARs, it is difficult to determine whether the copy-number dependency resulted from some specific property of the lysozyme gene or from an interaction between the gene and the MARs. The lack of copy-number-dependent expression in this experiment may reflect a less-than-perfect insulating ability of MARs or may be the consequence of MARs not flanking all WAP transgenes. Similar to the 7-kb mouse WAP transgenes, expression of a 4.2-kb rat WAP transgene in mice was position dependent and copy-number-independent (11). However, deleting 1.4 kb of 3′-flanking sequence from the rat WAP transgene resulted in high-level and copy-number-dependent expression (12, 20). This result may suggest that respective regulatory elements are located within 2.9 kb of WAP gene sequence but that their activity is modified by sequences outside this region. Alternatively, it may be that synergistic activity between regulatory elements may have been promoted by shortening the transgene (20). This view was supported by the single nonexpressing line that contained a single copy of the transgene (12, 20).

Although MARs appear to protect the WAP transgene from position effects in four insertion sites examined, low consti-

tutive expression throughout pregnancy and lactation was observed in a fifth line. Southern blot analysis of this line confirmed that WAP gene promoters were juxtaposed to MARs, but it also revealed rearrangements within the transgene locus. It is possible that this particular site of integration was not sufficiently buffered by MARs. A similar observation has been made with *scs* elements from a *Drosophila* heat shock gene (3). Constitutive expression may be caused by transcriptional regulation different from that encountered with the endogenous gene and the other transgenes. Consistent with this is the observation that the degree of tissue-specific transgene expression in mice carrying the human cytomegalovirus enhancer varied with the overall level of expression (21). The transgene in a low-expressing line was constitutively active in all tissues, and only a high-expressing line demonstrated marked tissue specificity.

Although interspersion of MARs into WAP transgene loci resulted in the establishment of domains of independent gene activity, we have no evidence that these MARs are actually boundaries of chromatin domains. However, because MARs exert their effects only in stably transfected cell lines (8) and in transgenic mice described here but do not exert effects upon transient transfection, MARs probably function at the chromatin level. Clearly chromatin boundaries and insulators are important in the informational organization of genetic material (22), and their removal can cause spillover of activation and deregulation (23). Both the chicken lysozyme MARs and the *scs* elements from a *Drosophila* heat shock locus appear to function as neutral boundaries and insulate heterologous genes from position effects. These effects clearly differ from the locus-control region of the human β-globin locus. The locus-control region can confer high-level expression, and it contains tissue-specific enhancers (24, 25). Consistent with the model that MARs create independent genetic domains are experiments in which purified MAR-binding protein can bind synergistically to widely separated sequences with the intervening DNA looping out (1). This result could cause the formation of topologically sequestered loop domains, in which WAP gene-regulatory elements can function without outside interference.

From a pragmatic point of view, significant benefits may be realized by including either homologous or heterologous MARs with transgene constructs used in transgenic livestock. Although construct dependent, the proportion of transgenic large animals that express their transgene is ≈60% (26, 27). Given that the cost of producing transgenic sheep and pigs is in the tens of thousands of dollars and production of transgenic cattle may be an order of magnitude higher, the use of MARs could substantially reduce production costs.

This paper is dedicated to Albrecht E. Sippel on the occasion of his 50th birthday.

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