# Hormone-induced modifications of the chromatin structure surrounding upstream regulatory regions conserved between the mouse and rabbit whey acidic protein genes

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The upstream regulatory regions of the mouse and rabbit whey acidic protein (WAP) genes have been used extensively to target the efficient expression of foreign genes into the mammary gland of transgenic animals. Therefore both regions have been studied to elucidate fully the mechanisms controlling WAP gene expression. Three DNase I-hypersensitive sites (HSS0, HSS1 and HSS2) have been described upstream of the rabbit WAP gene in the lactating mammary gland and correspond to important regulatory regions. These sites are surrounded by variable chromatin structures during mammary-gland development. In the present study, we describe the upstream sequence of the mouse WAP gene. Analysis of genomic sequences shows that the mouse WAP gene is situated between two widely expressed genes (*Cpr2* and *Ramp3*). We show that the hypersensitive sites found upstream of the rabbit WAP gene are also detected in the mouse WAP gene. Further, they encompass functional signal transducer and activator of transcription 5-binding sites, as has been observed in the rabbit. A new hypersensitive site (HSS3), not specific to the mammary gland, was mapped 8 kb upstream of the rabbit WAP gene. Unlike the three HSSs described above, HSS3 is also detected in the liver, but similar to HSS1, it does not depend on lactogenic hormone treatments during cell culture. The region surrounding HSS3 encompasses a potential matrix attachment region, which is also conserved upstream of the mouse WAP gene and contains a functional transcription factor Ets-1 (E26 transformation-specific-1)-binding site. Finally, we demonstrate for the first time that variations in the chromatin structure are dependent on prolactin alone.

Key words: mammary gland, prolactin, signal transducer and activator of transcription 5, whey acidic protein (WAP).

## INTRODUCTION

Whey acidic protein (WAP) has been described previously for the milk of the mouse [1], rat [2], rabbit [3], camel [4], pig [5], brushtail possum and Tammar wallaby [6]. Expression of the WAP gene is restricted to mammary-gland epithelial cells and is regulated by complex interactions between lactogenic hormones, extracellular matrix and intercellular interactions, as observed previously for other milk protein genes [2]. The hormones implicated in WAP gene expression have been defined in mouse primary organoid cells, where the induction of WAP gene expression depends mainly on the synergistic action of prolactin and glucocorticoids [7]. During the mid-pregnancy–lactation period, levels of WAP mRNA in rabbit increase by several thousand-fold. The high level of expression and the tissue specificity of the WAP gene render its upstream region a valuable tool to target a strong expression of heterologous genes into the mammary gland of transgenic animals. Upstream regions of the mouse [8,9] and rabbit [10] WAP genes have thus been linked to various genes. Although the levels of expression of foreign genes were among the highest described, they varied depending on both the length of the upstream region chosen and the gene that was expressed. Therefore the positions of the regulatory elements controlling this expression have been studied in both species to explain such differences. In our previous work, three DNase I-hypersensitive sites (HSSs) were located upstream of the rabbit WAP gene in the lactating mammary gland and were termed HSS0, HSS1 and HSS2 [11]. They correspond to three important functional regions described using WAP–chloramphenicol acetyltransferase (CAT) constructs transfected into rabbit primary mammary-gland cells [11]. All the hypersensitive sites in rabbit encompass a functional binding site for the signal transducer and activator of transcription 5 (Stat5), a known transcription factor implicated in milk-protein gene expression [12]. Furthermore, these results demonstrated that the sequences underlying these three rabbit hypersensitive sites could be aligned with three regions of the mouse WAP gene. We thus predicted important conserved regulatory regions in the mouse WAP gene. Although transgene expression containing the extensively used 6.3 kb upstream region of the rabbit WAP gene (which includes HSS0, HSS1 and HSS2) was always strong [10], this expression was not independent of the integration site, as has been observed also for the 2.4 and 4.1 kb mouse fragments [8,9]. These results suggest that further upstream regulatory elements may have a role to play in regulating the correct expression of the WAP gene.

The chromatin structure encompassing the rabbit hypersensitive sites varied during the mammary-gland cycle, but HSS2

Abbreviations used: BAC, bacterial artificial chromosome; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility-shift assay; Ets-1, E26 transformation-specific-1; HSS, DNase I-hypersensitive site; LCR, locus control region; MAR, matrix attachment region; PL, placental lactogen; Stat5, signal transducer and activator of transcription 5; tsp, transcriptional start point; WAP, whey acidic protein.

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The nucleotide sequences reported in this paper will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AF548632, AF548633 and BK000628 (TPA) for the mouse WAP gene and AY177248 for the rabbit WAP gene.

is the only hypersensitive site whose presence correlates with WAP gene expression [11]. These results suggest an important role for HSS2 in regulating the expression of this gene *in vivo*. A key step in elucidating the mechanism which controls WAP gene expression is to determine which hormones control this variable chromatin structure encompassing regulatory elements, more particularly around HSS2, upstream of the WAP gene. In sheep, placental lactogen (PL) II has been proposed as a potential inducer of modifications in the sheep $\beta$-lactoglobulin chromatin structure occurring during the first stage of lactogenesis [13], but few results are available to confirm this hypothesis. No PL has been described in rabbits, suggesting that other hormones are implicated in changes made to the chromatin structure surrounding the WAP gene during mammary-gland development. Unfortunately, induction of WAP expression has been detected only at low levels in the mammary-gland cell lines available [14]. The only model at our disposal to study the hormones responsible for the unique chromatin structure pattern observed during lactation is primary cell cultures.

At the time of the present study, new mouse and rabbit genomic sequences became available, and we extended our work to the more upstream region of the WAP gene. Sequence alignments between rabbit and mouse were performed and a new conserved region was identified, located between $-5266$ and $-5686$ from the mouse transcriptional start point (tsp). This conserved region, like two other conserved regions described previously, corresponded to an HSS. Using electrophoretic mobility-shift assay (EMSA), we demonstrated that each of these mouse HSS encompassed a functional Stat5-binding site, similar to findings in the rabbit. Furthermore, we mapped a new hypersensitive site (HSS3) at $-8$ kb from the rabbit tsp and detected, using EMSA, a functional transcription factor Ets-1 (E26 transformation-specific-1)-binding site in the vicinity of HSS3. Sequence analysis of the region surrounding HSS3 has allowed us to detect a potential matrix attachment region (MAR) that is also conserved in the mouse sequence. As more results became available for the rabbit model, variations in the chromatin structure surrounding rabbit hypersensitive sites were evaluated in rabbit primary mammary-gland culture cells. We thus demonstrate for the first time that these variations are dependent on prolactin alone.

## EXPERIMENTAL

### Animals

New Zealand White rabbits at day 20 of pregnancy were obtained from a slaughterhouse, in accordance with French Ministry of Agriculture guidelines (dated 19 April 1988). Lactating animals were killed within 1 h after suckling by their young. Female mice [(C57BL/6J $\times$ CBA)$F_1$] were killed on day 12 of lactation. The mammary gland and liver were dissected. In all the cases, the organs were dissected, deep-frozen in liquid nitrogen and then stored at $-20$ °C until use.

### Isolation of nuclei and DNase I digestion

Nuclei were isolated from freshly excised tissue or harvested rabbit primary mammary-gland cell cultures, and DNase I digestion was performed as described by Whitelaw and Webster [15]. Genomic DNA was purified by three cycles of phenol–chloroform extraction followed by ethanol precipitation. Purified DNA (10 $\mu$g) was digested using restriction enzymes, analysed on 1 % agarose gels and transferred to Nytran N™ membranes. The membranes were then hybridized with labelled probes as described in the Figure legends. Probes were labelled by random priming using [$\alpha$-$^{32}$P]dCTP (Amersham Biosciences, Orsay, France). The specific activity of probes was of the order of $10^9$ c.p.m./$\mu$g. Radioactive signals were detected using STORM™ and analysed with ImageQuant™ software.

### EMSA

Double-stranded probes end-labelled with [$\gamma$-$^{32}$P]ATP and the bacteriophage T4-induced polynucleotide kinase (50000 c.p.m.) were incubated with 5 $\mu$g of nuclear extract for 30 min at room temperature (24 °C), as described previously [11]. In some cases, nuclear extracts were preincubated for 30 min at room temperature with a 3–10-fold excess of unlabelled probe or with 1 $\mu$l of specific monoclonal antibodies raised against mouse Stat5a (reference no. 13-3600, Zymed Laboratories, San Francisco, CA, U.S.A.; reference no. 06 553, Upstate Biotechnology, Lake Placid, NY, U.S.A.), Stat5b (reference no. 13-5300, Zymed Laboratories; reference no. 06 554, Upstate Biotechnology) or Ets-1 (reference no. Sc-350 X, Santa Cruz Biotechnology, Heidelberg, Germany) as described in the Figure legends. After the preincubation and incubation periods, samples were loaded on to a 5 % (w/v) non-denaturing polyacrylamide gel in 12.5 mM Tris (pH 8.3), 12.5 mM boric acid and 0.25 mM EDTA and subjected to electrophoresis at 200 V for 2 h. Gels were then transferred to a Whatman DE81 membrane and dried under vacuum. Radioactive signals were detected using STORM™ and analysed with ImageQuant™ software.

### Northern-blot analysis

Total RNA was extracted from the mammary glands, analysed on 1 % agarose gel, transferred to Nytran and hybridized with an $\alpha$-$^{32}$P-labelled WAP probe, as described previously [10]. Radioactive signals were detected using STORM™ and quantified with ImageQuant™ software.

### Cell cultures and hormone inductions

Organoids were isolated from the mammary gland of a 20-day-pregnant rabbit and seeded on 100 mm plastic dishes as described previously [11]. The medium was changed 2 days later. Hormones were added the following day as mentioned in the Figure legends [5 $\mu$g/ml insulin, 1 $\mu$g/ml prolactin, 500 ng/ml cortisol in 50 % (v/v) minimal essential medium, 50 % (v/v) F125 and 2 % (w/v) medium containing Ultroser SF]. Cells were harvested 24 h later in PBS. Jurkat cells were grown in suspension in an RPMI 1640 medium containing 10 % (w/v) foetal calf serus, 10 mM Hepes (pH 7.5), 1 mM sodium pyruvate, 38 mM D-glucose, 100 units/ml penicillin and 100 mg/ml streptomycin until a concentration of $10^7$ cells/ml was reached. The medium was changed every day. Cells were harvested by centrifugation.

### Sequence analysis

For the mouse WAP gene, genomic sequences were obtained from bacterial artificial chromosome (BAC)-WAP-218 clone [16] and confirmed and extended with sequences available from BAC RP23-169J19 clone (Mouse Ensembl Project; http://www.ensembl.org/Mus_musculus/). Sequence alignments and comparison analyses were performed using ALIGNn

(http://www.infobiogen.fr), PIPMaker (http://bio.cse.psu.edu/pipmaker/), RepeatMasker (http://ftp.genome.washington.edu/cgi-bin/RepeatMasker/) and *MacVector* (Accelrys). MARs were predicted using MAR-Wiz (http://www.futuresoft.org).

## RESULTS

### A potential regulatory region is located between − 5266 and − 5686 upstream of the mouse WAP gene

In a previous study, we identified three hypersensitive sites corresponding to important regulatory regions upstream of the rabbit WAP gene [11]. They were termed HSS0, HSS1 and HSS2 and are localized between − 0.5 and − 0.8, − 2.5 and − 3.2, and − 5.4 and − 5.8 kb respectively relative to the tsp. The sequence of these regions could be aligned with three regions of the mouse WAP gene (GenBank® accession no. MMWAPX1; extending from − 4120 to + 1), between − 530 and − 832, − 1281 and − 1407, and −1866 and −2304 bp relative to the tsp. These conserved regions encompassed potential Stat5-binding sites in the mouse as in the rabbit. In this regard, using the MMWAPX1 sequence, we could predict important regulatory regions upstream of the mouse WAP gene.

New insights into the mouse genome provided access to sequences previously unknown. We have compiled the full sequence of the 13 kb upstream region of the mouse WAP gene, included within a BAC clone (BAC-WAP-218, 23990 bp [16]; GenBank® accession no. BK000628; Figure 1) in combination with mouse sequences available from the Mouse Ensembl Project (BAC RP23-169J19; http://www.ensembl.org/Mus_musculus/). Mouse and rabbit sequence alignments were performed to extend our previous results.

Two genomic sequences were available which corresponded to the mouse WAP gene: sequence U38816, entirely included within BK000628 (Figure 1), and MMWAPX1. Discrepancies were detected between the MMWAPX1 and BK000628 sequences. The BK000628 fragment encompassed the entire MMWAPX1 fragment, which seemed to lack a central 3.4 kb region at − 1.6 kb from the tsp (Figure 2A). Restriction maps deduced from the two sequences were prepared and Southern-blot experiments were performed (Figure 2B) to analyse such discrepancies. Mouse [(C57BL/6J × CBA)$F_1$] mammary-gland DNA was digested with *Bst*EII or *Bam*HI (Figure 2A), blotted and probed with the complete mouse WAP cDNA probe [17]. When digested with *Bam*HI (Figure 2B, lane 0), we detected an 11 kb fragment, as expected from the BK000628 restriction map. When DNA was digested with *Bst*EII (Figure 2B, lane 1), a 9.3 kb fragment was detected, in good agreement with the BK000628 sequence, and not a 5.9 kb fragment as expected from MMWAPX1. Based on this result, we used the BK000628 sequence as the new reference in the present study, since its restriction map corresponds to the genomic DNA of the mouse strain [(C57BL/6J × CBA)$F_1$] used in our laboratory. Sequence alignments between the rabbit (X52564) and mouse (BK000628) 10 kb upstream regions of the WAP gene were performed (ALIGNn). Four regions were found to be conserved between these two species, extending from + 1 to − 200, − 530 to − 832, − 1281 to − 1368 and − 5266 to − 5686 bp in the mouse, with similarities ranging from 58.3 to 69.7 % (Figure 3). The first conserved region encompassed 200 bp immediately upstream of the mouse WAP gene. The other three conserved regions encompassed the corresponding rabbit hypersensitive sites described above, as expected from our previous work. The more distal region, corresponding to HSS2 in the rabbit WAP gene, is now located between − 5266 and − 5686 bp upstream of the mouse WAP gene.

### Four hypersensitive sites are detected upstream of the mouse WAP gene

The sequence alignments described above allowed us to extrapolate similar positions of important rabbit regulatory

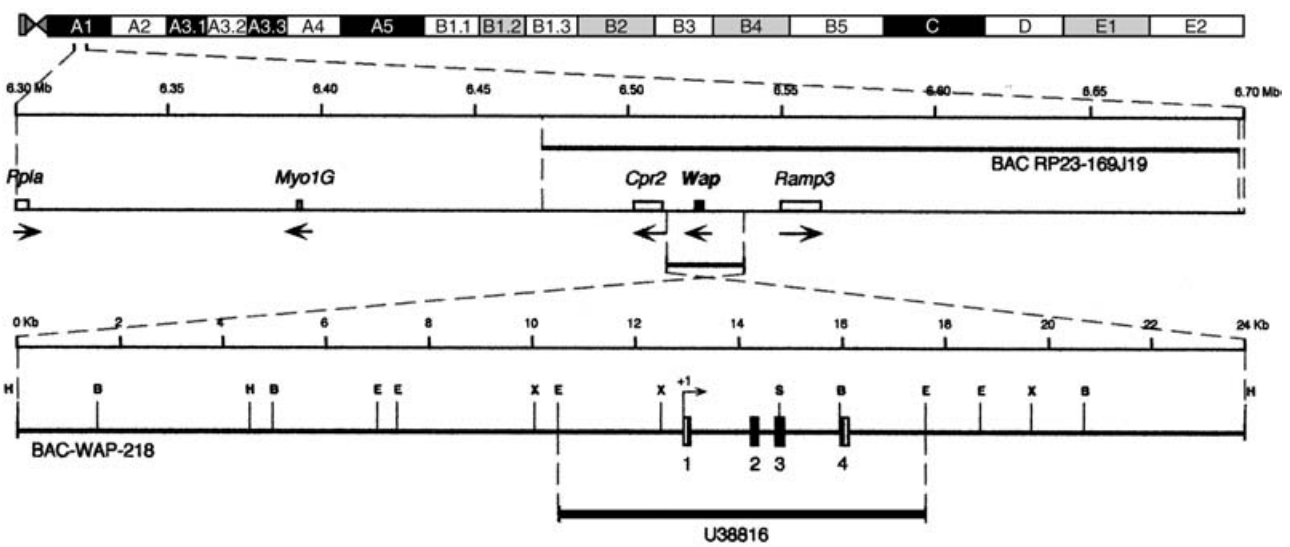


**Figure 1 Schematic representation of the genomic region surrounding the mouse WAP gene**

(From top to bottom): mouse chromosome 11, according to the Mouse Ensembl Database (http://www.ensembl.org/Mus_musculus/); an expanded 400-kb-long region from the A1 band of mouse chromosome 11, from nucleotide positions 6.3–6.7 Mb, centred around the WAP gene (the relative position of the BAC RP23-169J19 clone, used in the present study, is indicated); the restriction map of the mouse BAC-WAP-218 clone, 23990 bp [16] (GenBank® accession numbers TPA BK000628, AF548632 and AF548633); and the previously reported mouse WAP genomic sequences (GenBank® accession number U38816). Horizontal arrows indicate the transcriptional direction of genes. Exons are shown as boxes and translated regions are indicated as solid boxes, + 1 indicates the tsp of the mouse WAP gene. Abbreviations: *Ppia*, peptidyl-prolyl *cis–trans* isomerase A gene; *Myo1G myosin-1G*, fragment predicted Ensembl gene; *Cpr2*, hypothetical 10.5 kDa protein, similar to the human gene for cell cycle progression 2 protein; *Ramp3*, receptor-calcitonin-activity-modifying protein 3 gene. Restriction enzymes: H, *Hin*dIII; B, *Bam*HI; E, *Eco*RI; X, *Xho*I; S, *Sal*I.

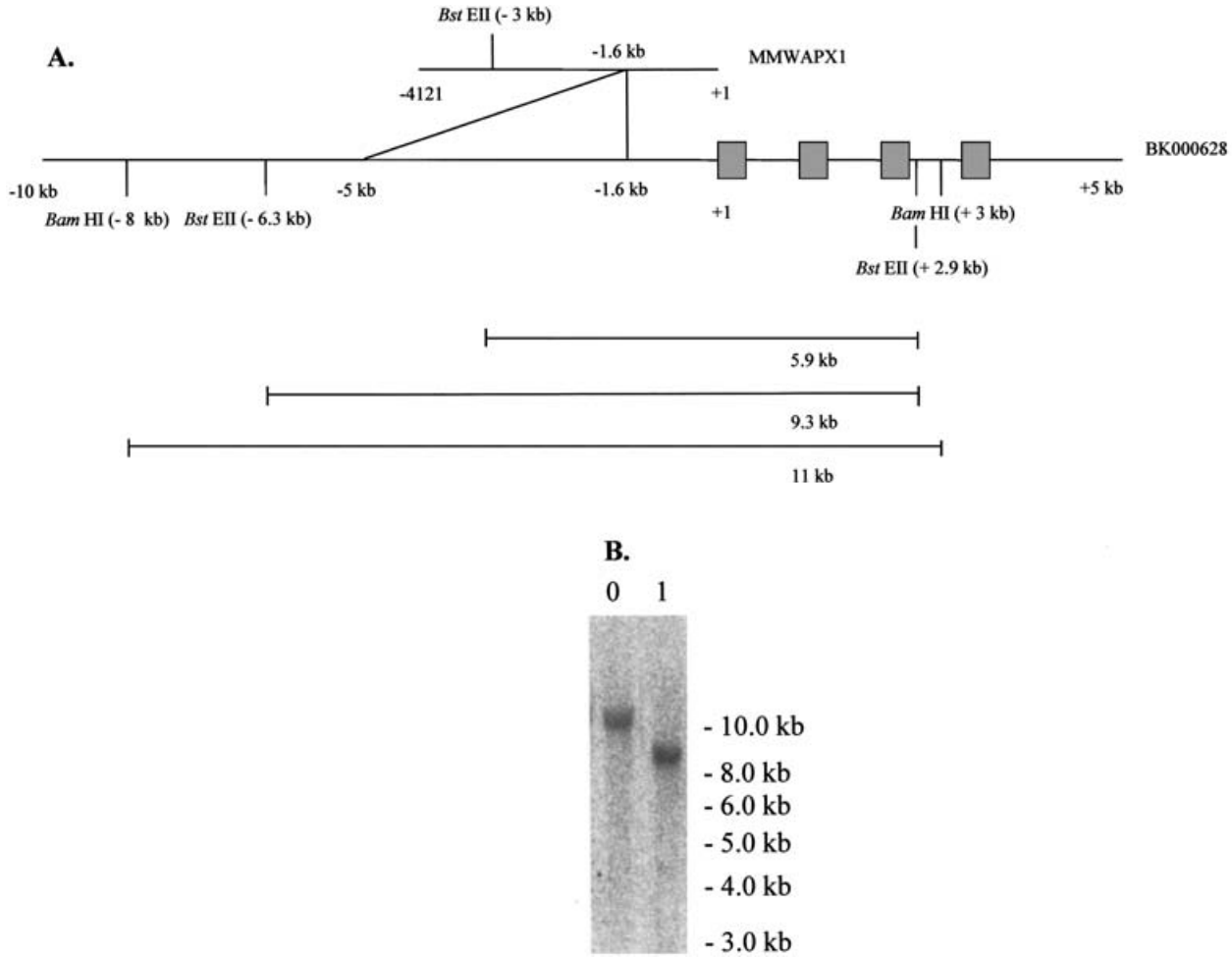


**Figure 2 Restriction map analysis of mouse MMWAPX1 and BK000628 sequences**

(**A**) Restriction map of the MMWAPX1 and BK000628 fragments. Restriction fragments generated after digestion with *Bam*HI or *Bst*EII are indicated by horizontal lines. Exons are shown as grey boxes. (**B**) Genomic mouse mammary-gland DNA was digested with *Bam*HI (lane 0) or *Bst*EII (lane 1), subjected to gel electrophoresis and analysed by Southern blotting, hybridizing with the complete mouse WAP cDNA probe [17].

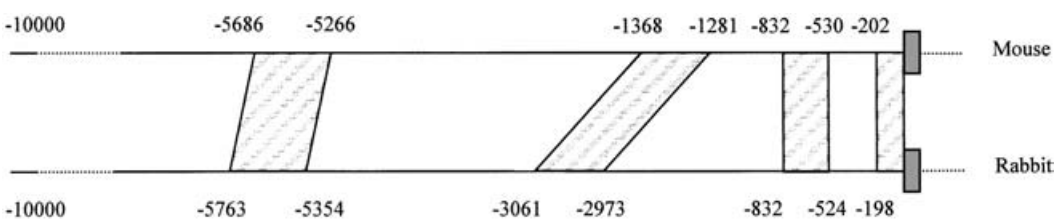


**Figure 3 Sequence alignments of the 10 kb upstream regions of the mouse and rabbit WAP genes**

Sequence alignments of the 10 kb upstream regions of the mouse and rabbit WAP genes were performed using ALIGNn. Conserved sequences between the rabbit and mouse are depicted by hatched boxes. The first exon is shown as a grey box.

regions to the mouse WAP gene. To characterize the chromatin structure encompassing the upstream region of the mouse WAP gene, indirect end-labelling experiments were performed using DNase I. Nuclei isolated from lactating mouse mammary glands were subjected to mild DNase I digestion. DNA was then extracted and digested with appropriate restriction enzymes, and the resulting fragments were analysed on Southern blots. The probes used in these experiments were located at the 5′- or 3′-ends of the restriction fragment encompassing the expected hypersensitive sites. When DNA was digested with *Bgl*II (Figure 4A), the major band detected was located at 3.4 kb, as expected (Figure 4B, lane 0). After mild DNase I treatment, several other fainter bands of lower molecular masses were detected at 1.9 and 2.4 kb (Figure 4B, lanes 1 and 2). They corresponded to two hypersensitive sites, located around the tsp and at $-0.5$ kb from it. They were termed tsp and HSS0

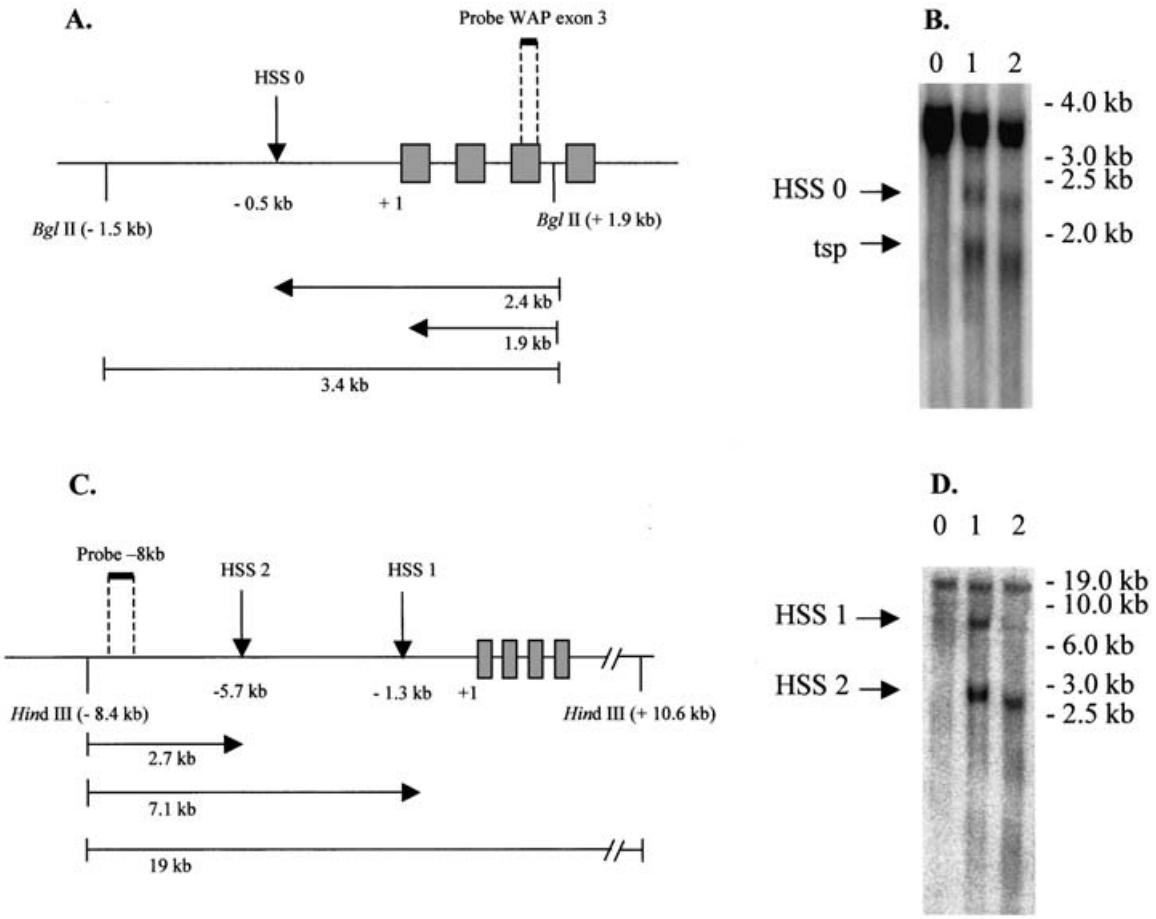


**Figure 4 Detection of hypersensitive sites in the upstream region of the mouse WAP gene in the lactating mammary gland**

(**A**, **C**) Restriction maps of the upstream region of the mouse WAP gene. HSSs are indicated on the map by vertical arrows and fragments generated by DNase I are indicated by horizontal arrows. Exons are shown as grey boxes. (**B**, **D**) Characterization of DNase I hypersensitivity in mouse lactating mammary gland. Nuclei were incubated for 0, 1 or 3 min (lanes 0, 1 and 2 respectively) with DNase I. DNA was digested by *Bgl*II (**B**) or *Hin*dIII (**D**), subjected to gel electrophoresis and blotted. Generated fragments were detected with a probe specific to the WAP exon 3 (3′-end of the *Bgl*II fragment) or with the − 8 kb probe (5′-end of the *Hin*dIII fragment). Positions of the HSSs are indicated by arrows on the left-hand side of the blots.

respectively. Using *Hin*dIII (Figure 4C) as the restriction enzyme and using a probe located at the 5′-end of the generated restriction fragment, we detected two other hypersensitive sites further upstream of the mouse WAP gene (Figure 4D, lanes 1 and 2). They were termed HSS1 and HSS2 and mapped at − 1.3 and − 5.7 kb from the tsp respectively. None of these hypersensitive sites was detected in mouse liver, which does not express the WAP gene (results not shown). Therefore hypersensitive sites are located in genomic regions conserved between the rabbit and the mouse WAP genes.

### HSS0, HSS1 and HSS2 encompass a functional Stat-binding site

The sequence of the 13 kb upstream region of the mouse WAP gene was scanned for transcription factor potential binding sites using TFSEARCH. As observed previously for the upstream region of the rabbit WAP gene, several potential binding sites for different transcription factors (CCAAT-enhancer-binding protein, c-Ets and Stat5) were detected. Among them, seven potential binding sites exhibited some degree of similarity to the Stat5 consensus, a transcription factor known to be implicated in milk-protein gene expression [12]. Only three of these potential binding sites, located between − 570 and − 561, − 1338 and 1329, and − 5556 and − 5547 bp relative to the tsp, displayed more than 90 % similarity to the Stat5 consensus. These sites were located within HSS0, HSS1 and HSS2 respectively. We therefore investigated whether Stat5 was capable of binding *in vitro* to these three sequences. EMSAs were performed using $^{32}$P-radiolabelled double-stranded oligonucleotides corresponding to the potential Stat5 sequences (Table 1, probes 0–2) incubated with lactating mouse mammary-gland nuclear extracts. After incubation with

**Table 1 Sequences of the oligonucleotides used in EMSA**

Double-stranded oligonucleotides used in bandshift assays are listed. The localizations of the Stat5 or Ets-1 potential binding sites relative to the mouse or rabbit tsp are given in parentheses.

| Type of probe | Sequence |
|---|---|
| Probe 0 (− 561 to − 570) | 5′-CCTTCTGGGAAGTTGGC-3′<br>3′-GAAGACCCTTCAACCGG-5′ |
| Probe 1 (− 1329 to − 1338) | 5′-CCTTCTGGGAAACTCAA-3′<br>3′-GAAGACCCTTTGAGTTC-5′ |
| Probe 2 (− 5604 to − 5588) | 5′-CATTCCTAGAACCTTCT-3′<br>3′-TAAGGATCTTGGAAGAC-5′ |
| Probe 3 (− 7605 to − 7614) | 5′-CCTGCCCGGAAGTCAG-3′<br>3′-GACGGGCCTTCAGTCC-5′ |
| Probe C | 5′-GAGATTCTTAGAATTTAAA-3′<br>3′-CTCTAAGAATCTTAAATTT-5′ |

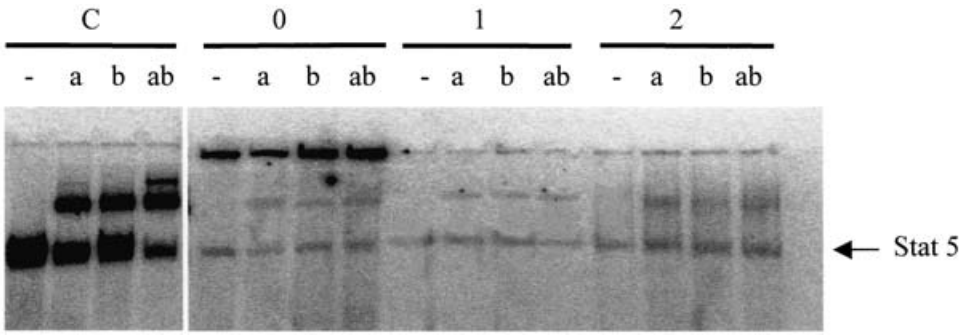


**Figure 5 EMSA analysis of Stat5 potential binding sites encompassing HSS0, HSS1 and HSS2 upstream of the mouse WAP gene**

Mouse lactating mammary-gland nuclear extracts were incubated with $^{32}$P-labelled oligonucleotide probes C and 0–2 (see Table 1) containing potential Stat5-binding site in the absence (–) or presence of Stat5a (a), Stat5b (b) or both Stat5a and Stat5b (ab), antibodies as indicated. The arrow indicates the position of the complex containing Stat5.

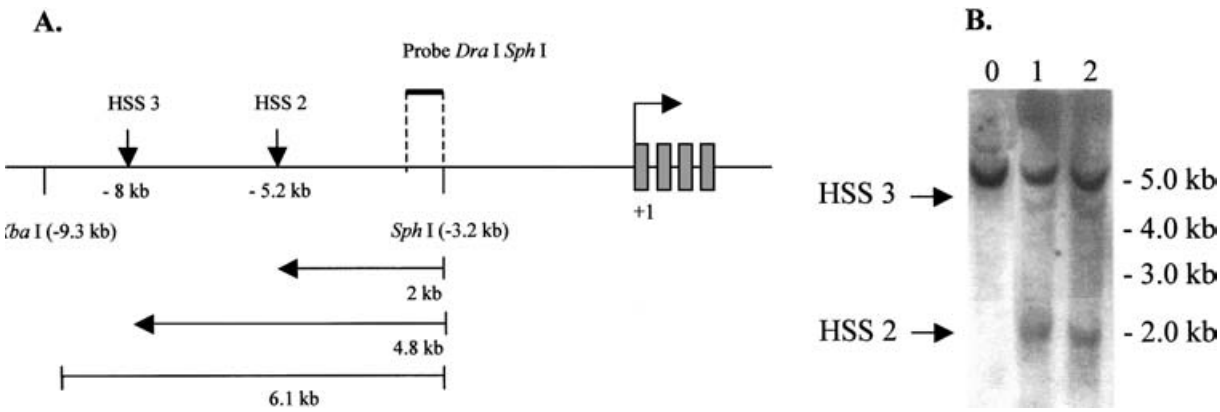


**Figure 6 A new hypersensitive site detected far upstream of the rabbit WAP gene in the lactating mammary gland**

(**A**) Restriction map of the upstream region of the rabbit WAP gene. HSSs are indicated on the map by vertical arrows and fragments generated by DNase I are indicated by horizontal arrows. Exons are shown as grey boxes. (**B**) Characterization of DNase I hypersensitivity in rabbit lactating mammary gland. Nuclei were incubated for 0, 1 or 3 min (lanes 0–2 respectively) with DNase I. DNA was digested by *Xba*I and *Sph*I, subjected to gel electrophoresis and blotted. Generated fragments were detected with a WAP-specific *Dra*I–*Sph*I probe, at the 3′-end of the restriction fragment. Positions of the HSSs are indicated by arrows on the left-hand side of the blot.

nuclear extracts, each probe was capable of forming a complex (Figure 5, panels 0–2, lanes indicated by '–') which displayed an electrophoretic mobility similar to that of the Stat5-specific complex observed with the reference probe C, designed using the proximal $\alpha$s1 casein promotor (Figure 5, panel C). Signals were however much fainter using WAP probes when compared with those obtained using the reference probe C, most probably because we used shorter probes to avoid non-specific interactions with flanking sequences. Two isoforms of Stat5, Stat5a and Stat5b have been described in the mouse, and monoclonal antibodies are available. When these antibodies were used separately or together, the complex was partially shifted (Figure 5, panels 0–2, lanes a, b and ab), as was the case for the reference probe C (Figure 5, panel C, lanes a, b and ab). No complex was observed when probes were not incubated with mouse mammary-gland nuclear extracts (results not shown). We concluded that HSS0, HSS1 and HSS2 all encompass a sequence capable of interacting with activated Stat5a and Stat5b *in vitro*.

### A new distal hypersensitive site is detected upstream of the rabbit WAP gene

When the rabbit 6.3 kb upstream region of the WAP gene was used to control foreign gene expression in the mammary gland of transgenic animals, transgene expression was not independent of the integration site of the transgene [10]. Thus we searched for new regulatory elements far upstream of the rabbit WAP gene, using the same indirect end-labelling technique using DNase I. Nuclei isolated from the lactating rabbit mammary gland were submitted to mild DNase I digestion. DNA was then extracted and digested by *Xba*I and *Sph*I (Figure 6A). Using a probe located at the 3′-end of the restriction fragment, we were able to detect two hypersensitive sites at – 5.2 bp and approx. – 8 kb, relative to the tsp (Figure 6B, lanes 1 and 2). The first hypersensitive site corresponded to HSS2, which has been described previously. The second was a new hypersensitive site located far upstream of known rabbit HSSs and it was termed HSS3. The signal observed for HSS3 was weaker when compared with that of HSS2 (Figure 6B, lanes 1 and 2). This result could be explained by HSS2 being located between the probe and HSS3. Furthermore, HSS3 was also detected in the pregnant and post-weaning rabbit mammary gland and in the liver (Figures 7B, 7C and 7E respectively). Therefore HSS3 is not specific to the mammary gland.

### The region surrounding HSS3 encompasses a functional Ets-1-binding site

The sequence surrounding HSS3 (GenBank® accession no. AY177248) was also scanned with TFSEARCH for known

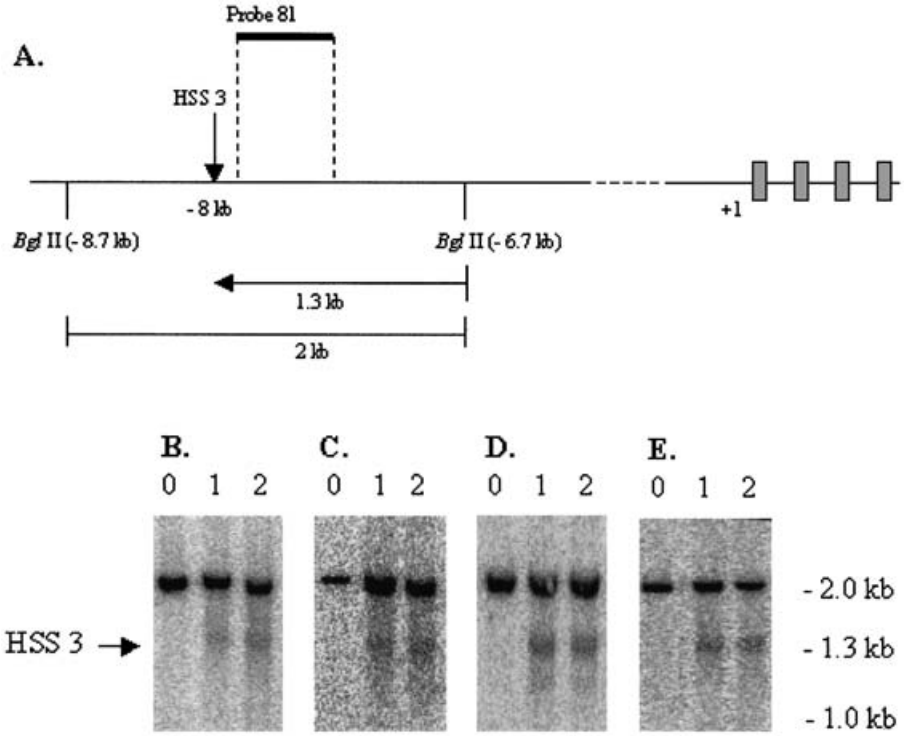


**Figure 7 HSS3 detected far upstream of the rabbit WAP gene in the liver and the mammary gland at all stages of development**

(**A**) Restriction map of the upstream region of the rabbit WAP gene. HSS is indicated on the map by a vertical arrow, the fragment generated by DNase I is indicated by a horizontal arrow. Exons are shown as grey boxes. (**B**–**E**) Characterization of DNase I hypersensitivity in the liver (**B**) and in the mammary gland during pregnancy (**C**), lactation (**D**) and after weaning (**E**). Nuclei were incubated for 0, 1 or 3 min (lanes 0, 1 and 2 respectively) with DNase I. DNA was digested by *Bgl*II, subjected to gel electrophoresis and blotted. Generated fragments were detected with a WAP-specific probe 81. The position of the HSS is indicated by the arrow on the left-hand side of the blot.

transcription factor potential binding sites. A potential Stat5-binding site was found at − 7617 bp from the tsp. This site also overlapped a GGAA core motif characteristic of the binding site for Ets-related proteins. These proteins share the Ets domain, a unique DNA-binding domain [18]. The potential Stat5-binding site displayed less similarity (84 %) to the described consensus and was unable to interact with Stat5 *in vitro* (results not shown). EMSA was performed to determine whether Ets-1 was capable of binding to this potential sequence. Several complexes were able to bind to probe 3 (Table 1) encompassing the potential Ets-1-binding site (Figure 8, lane 0). One complex formed in the presence of probe 3 was totally shifted with a monoclonal Ets-1-specific antibody (Figure 8, lane1). The supershift signal was stronger than the original shift, as has been observed previously for other antibodies [19]. We thus concluded that the region surrounding HSS3 encompasses a functional Ets-1-binding site.

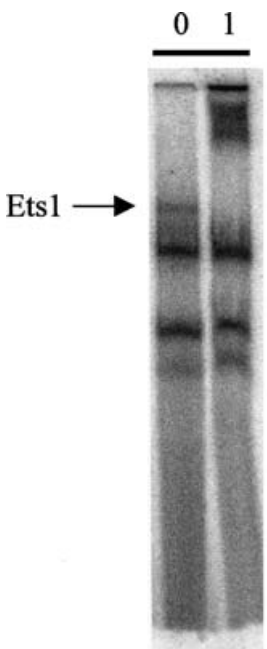


**Figure 8 EMSA analysis of the Ets-1 potential binding sites encompassing HSS3 upstream of the rabbit WAP gene**

Rabbit lactating mammary-gland nuclear extracts were incubated with $^{32}$P-labelled oligonucleotide probe 3 containing a potential Ets-1-binding site in the presence (lane 1) or absence (lane 0) of antibody raised against Ets-1. The arrow indicates the position of the complex containing Ets-1.

### The region surrounding HSS3 encompasses a potential MAR

Analysis of the sequences surrounding HSS3 revealed that it is an AT-rich region. MARs are known to contain a high percentage of AT in their sequences. MARs have been shown to participate in the insulation of transcription elements from the surrounding chromatin. The 10 kb upstream region of the rabbit WAP gene was scanned with MAR-Wiz (http://www.futuresoft.org), an informatic tool designed to detect the presence of MARs, which has proved its usefulness in other expression domains [20]. One region that displayed a relevant score was centred at approx. − 7 kb from the tsp (Figure 9A). The same analysis was performed using the upstream region of the mouse WAP gene.

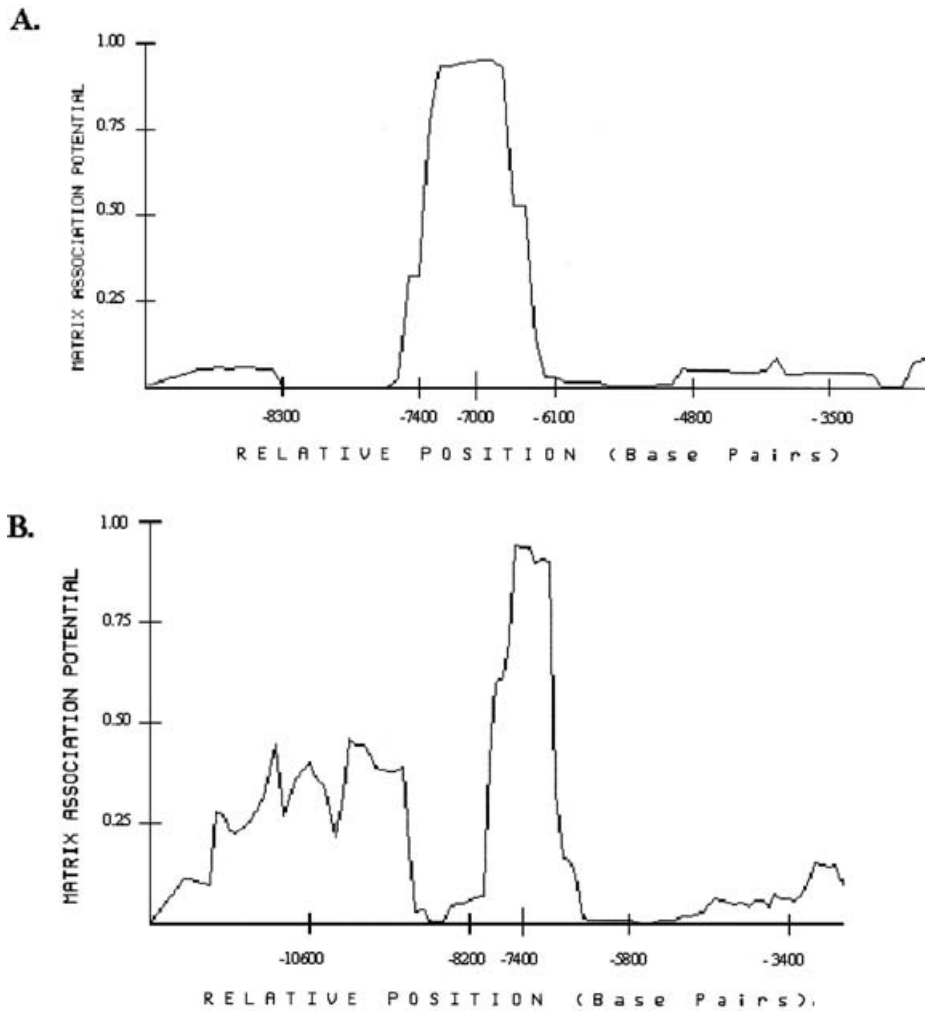


**Figure 9 Informatic prediction of MAR elements in the upstream 5′ regions of the rabbit and mouse genes**

Graphical output from the MAR-Wiz program (http://www.futuresoft.org), depicting the MAR-potential over rabbit (**A**) and mouse (**B**) at the upstream 5′ region of WAP sequences. Nucleotide positions, relative to the corresponding tsp, are indicated. Two major peaks are shown, at − 7 kb in the rabbit WAP gene and at − 7.4 kb in the mouse WAP gene.

As for the rabbit WAP gene, only one region displayed a relevant score. This region was centred at approx. − 7.4 kb from the tsp (Figure 9B). We concluded that a potential MAR exists between − 7 and − 8 kb upstream of both the rabbit and mouse WAP genes.

### Variations in the chromatin structure surrounding the rabbit hypersensitive sites are dependent on prolactin alone

Four hypersensitive sites are located in the 10 kb upstream region of the rabbit WAP gene. Whereas HSS1 and HSS3 were detected at all stages of the mammary-gland cycle, the chromatin structure surrounding HSS0 and HSS2, as probed by DNase I hypersensitivity, varied between pregnancy, lactation and after weaning. HSS0 was present during lactation and after weaning. HSS2, detected only during lactation, was the only site whose presence correlates with the WAP gene expression [11]. These results suggest a key role for HSS2 in regulating the WAP gene expression. The hormones responsible for variations in the chromatin structure surrounding the WAP gene remain unknown. Using rabbit primary mammary-gland cell cultures, treated with or without various hormones, we determined the hormones which were essential for establishing a chromatin structure that allowed WAP gene expression. Nuclei were isolated from rabbit primary mammary-gland cells treated with or without lactogenic hormones (cortisol and/or prolactin). They were exposed to mild DNase I treatment. DNA was then isolated, digested with *Bgl*II (Figure 10A) and analysed on Southern blots with probe 31, specific to the 5′-end of the restriction fragment. The main signal was observed for a 5 kb restriction fragment, as expected (Figures 10B–10E, lanes 0). Mild DNase I treatment induced the appearance of several smaller bands. Independent of the hormone treatment, an additional sharp band corresponding to a 3.5 kb fragment was observed (Figures 10B–10E, lanes 1 and 2). This corresponded to HSS1 described previously. When prolactin was used to stimulate the cell cultures, several additional diffuse bands, corresponding to 0.9–1.2 kb fragments, were also detected (Figure 10D, lanes 1 and 2). These bands were not detected when the cells were cultured in the presence of cortisol alone (Figure 10C, lanes 1 and 2). All these smaller fragments were due to the digestion of a DNase I-hypersensitive region, previously termed HSS2. Cortisol is known to amplify the prolactin effect on WAP gene expression [21]. As expected from the above results, HSS2

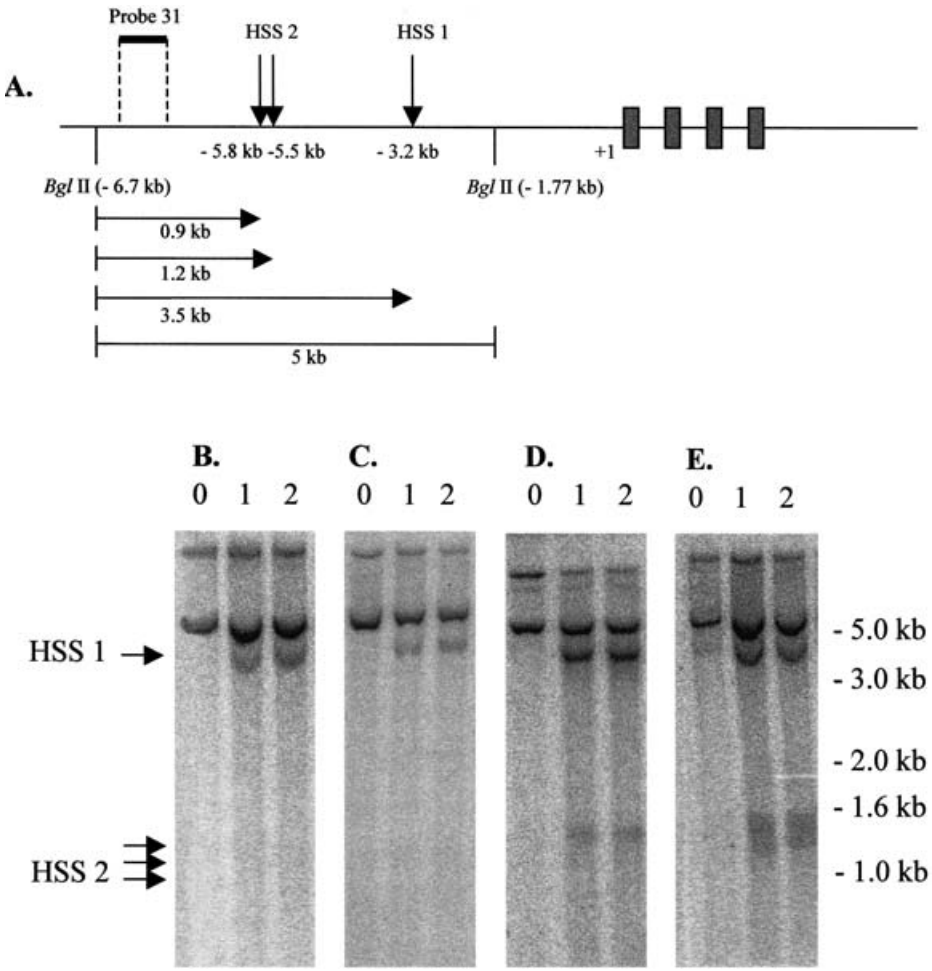


**Figure 10 Detection of hypersensitive sites in the upstream region of the rabbit WAP gene in rabbit primary mammary-gland cell cultures**

(**A**) Restriction map of the upstream region of the rabbit WAP gene. HSSs are indicated on the map by vertical arrows and fragments generated by DNase I are indicated by horizontal arrows. Exons are shown as grey boxes. Nuclei isolated from rabbit primary cell culture, either untreated (**B**) or treated with cortisol (**C**), prolactin (**D**) or cortisol and prolactin (**E**), were incubated for 0, 1 or 2 min (lanes 0, 1 and 2 respectively) with DNase I. DNA was digested with *Bgl*II, subjected to gel electrophoresis and blotted. Generated fragments were detected with the probe 31. The positions of the HSSs are indicated by arrows on the left-hand side of the blot.

was also detected when cortisol and prolactin were introduced together in the cell culture medium. The appearance of HSS2 was thus dependent on the presence of prolactin alone in the culture medium, whereas HSS1 was maintained after 5 days of culture in the absence of prolactin. Similar analyses were performed for HSS3 and HSS0. As expected from our *in vivo* data, we detected HSS3 independent of hormone treatments. HSS0 was only present after prolactin treatments, as described for HSS2 (results not shown). We therefore concluded that the presence of HSS0 and HSS2 was dependent on prolactin, whereas HSS1 and HSS3 were not dependent on the continuous presence of prolactin.

## DISCUSSION

The results described above identify a new conserved region located between $-5266$ and $-5686$ at the mouse WAP gene tsp and corresponding to the rabbit HSS2. This result may be surprising, since the mouse equivalent of rabbit HSS2 had been located previously between $-1866$ and $-2304$ bp relative to the mouse WAP tsp. However, such discrepancies could be explained by the differences encountered between a previously reported mouse WAP upstream sequence (MMWAPX1) and the sequence BK000628 reported in the present study and this is in agreement with the mouse WAP sequences available from the Mouse Ensembl Project. Interestingly, under each rabbit hypersensitive site, we were able to identify functional Stat5-binding sites in both mouse and rabbit sequences. Sequences from these functional binding sites display a high degree of similarity to the Stat5 consensus described previously ($> 90\,\%$). Four other potential binding sites were detected outside the conserved regions in the 10 kb upstream region of the mouse WAP gene, but they displayed only slight similarity to the known consensus ($< 85\,\%$). In the $\beta$-lactoglobulin promoter, three functional Stat5-binding sites have also been detected and they have been shown to act in co-operation to achieve maximum expression of this gene [12]. Our results suggest that in the WAP gene (as in the $\beta$-lactoglobulin gene), multiple Stat5-binding sites act in co-operation to control milk-protein gene expression.

The three mammary-gland-specific HSSs (HSS0, HSS1 and HSS2) identified in the lactating mouse mammary gland encompassed the three regions conserved between the rabbit and mouse WAP genes. Interestingly, these are the only conserved regions in the 10 kb upstream sequence of the WAP gene. HSS0

and HSS2 were detected at the same locations in the mouse and rabbit WAP genes. However, HSS1 was mapped more proximally in the mouse WAP gene when compared with the rabbit WAP gene. The differences observed (1.6 kb) could be explained by the presence of several repetitive sequences differing between the two species. We concluded that the hypersensitive sites found upstream of the WAP gene are conserved in the mouse and rabbit mammary glands. Such inter-species conservation has not been observed for the goat and sheep $\beta$-lactoglobulin genes, where a different hypersensitive pattern was detected in each species [22]. Identification of a fourth hypersensitive site (tsp) located around the tsp was not unexpected, as transcription complexes assembled on the TATA box may alter the chromatin structure in this region. In the rat WAP gene, a hypersensitive site has also been detected around the tsp, at $-100$ pb [23]. A similar proximal hypersensitive site has also been detected in the sheep $\beta$-lactoglobulin gene and its importance has been demonstrated using 5′ resected gene constructs [24]. Furthermore, several transcription-factor-binding sites have been identified previously within the WAP proximal promotor region. Using mouse WAP–CAT constructs containing 2.5 kb of the 5′-flanking region of the WAP gene stably transfected into the mouse mammary epithelial cell line, HC11, important regulatory regions for transgene expression have been localized between $-2500$ and $-1500$ and between $-450$ and the initiation site [25]. These results correlate with the localization of HSS described above. To date, the mouse 2.4 and 4.1 kb and rabbit 6.3 kb upstream regions of the WAP gene have been used extensively in transgenic experiments with different degrees of success [8–10]. The mouse WAP fragments do not include HSS2, whereas the 6.3 kb upstream region of the rabbit WAP gene includes this crucial hypersensitive site. These results would explain the reported differences in expression levels and patterns observed between mouse and rabbit WAP constructs. The incorporation of all relevant HSSs found in the mouse or rabbit WAP gene and reported in the present study, for subsequent transgenic approaches (as in the case of BAC-WAP-218-derived transgenic constructs), has resulted in the elevated and faithfully controlled expression of heterologous genes [26] (T. Mata and L. Montoliu, unpublished work). It is generally accepted that the use of large genomic sequences (i.e. BAC or yeast artificial chromosome) usually guarantees the strong and position-independent expression of heterologous constructs in transgenic animals [27]. However, the molecular basis for such a good performance is not often well understood. In the present study, we provide evidence, at the chromatin and cellular levels, recommending the inclusion of all these reported HSSs in future transgenic experiments based on WAP-derived heterologous constructs.

We mapped a new rabbit hypersensitive site (HSS3) at $-8$ kb from the tsp. HSS3 was detected at all stages of the mammary gland. The maintenance of HSS3 did not require the presence of lactogenic hormones. Moreover, HSS3 was also present in the liver, unlike HSS02, which were only detected in the mammary gland. Such a non-cell-specific HSS has been described previously in the human $\beta$-globin locus control region (LCR) [28] and it is believed to participate in the LCR function [29]. In transgenic flies and mice, other *cis*-acting DNA regions called MARs, which contain both cell-specific and non-cell-specific HSSs [30], have been shown to allow constructs to be transcriptionally active, independent of the site of integration [20,31]. Such LCR or MAR elements are yet to be described in rabbit or mouse WAP genes. During transgenic experiments, the rabbit (6.3 kb) or mouse (2.4 and 4.1 kb) upstream WAP fragments were unable to control transgene expression independent of the integration site [8,10]. However, when linked to the MAR fragment from the chicken lysozyme gene, the resulting constructs have been shown to exhibit improved expression frequency in some cases [32] and were insulated from position effects [31]. Sequence analysis of the region surrounding HSS3 allowed us to detect a potential MAR in the mouse and rabbit WAP upstream sequences. This MAR is thus localized in the vicinity of the non-mammary-gland-specific HSS3 in both species, at $-7$ kb in the rabbit WAP gene and at $-7.4$ kb in the mouse WAP gene. In the mouse genome, this MAR element is also located at 18.8 kb from a neighbouring gene, *Ramp3*, a ubiquitously expressed gene encoding a member of the Ramp family, which is required to transport calcitonin-like receptors to the plasma membrane [33] (Mouse Ensembl Project, see Figure 1). It is expected that it will be in the same position in the rabbit. The putative MAR we detected may contribute to insulating the WAP gene, whose expression is restricted to the mammary gland, from the influence of the neighbouring, ubiquitously expressed *Ramp3* gene. Similarly, another widely expressed gene, *Cpr2*, encoding a cell-cycle-regulatory protein, has been recently located 9.5 kb downstream of the mouse WAP gene [34] (Mouse Ensembl Project, see Figure 1). The relative position of *Ramp3* and *Cpr2* genes is conserved in the human genome (human chromosome 7, Human Ensembl Project) where no WAP-like genes have been identified to date. Probably, a similar genomic structure would be encountered in other mammals, such as rabbits. MAR sequences have also been detected in the downstream 3′ region of the mouse WAP gene, where they may function as potential boundary elements, insulating the tissue-specific expression of the WAP gene from the generalized expression of the neighbouring *Cpr2* gene. These 5′- and 3′-MAR elements of the mouse WAP expression domain are included in the BAC-WAP-218 clone and may explain its performance in transgenic mice (T. Mata and L. Montoliu, unpublished work).

Furthermore, we detected a functional Ets-1-binding site in the vicinity of HSS3 and the potential MAR. The Ets transcription factor family is involved in a variety of mammalian developmental processes at the cellular, tissue and organ levels. It is implicated in cellular proliferation, differentiation, migration, apoptosis and cell–cell interactions [35]. A functional binding site for Ets-1 has been located between $-114$ and $-105$ bp in the mouse, rat and rabbit WAP genes. Ets-1, binding to this site, has been shown to contribute to selective activation of WAP transgenes during pregnancy, but Ets-1 is not required for its high activity during lactation [36,37]. The functional Ets-1-binding site described in the present study may also contribute to control the WAP gene expression during pregnancy. Since Ets-1 is a member of the family of Ets transcription factors that recognize a similar sequence, it is probable that the sequence detected could bind to any member of this family. For example, polyoma enhancer activator 3, the founding member of a subfamily of closely related Ets genes that includes the ETS variant gene 1 (ETSV1) and the ETS variant gene 5 (ERM), has been demonstrated to play a key regulatory role in both mammary-gland development and oncogenesis [38]. Taken together, our results suggest that the region encompassing HSS3, the functional Ets-1-binding site and the potential MAR may play a different role in WAP gene regulation when compared with the role of HSS0, HSS1 and HSS2. Among other possibilities, as discussed above, this specific role may be related to the establishment of the 5′ border of the WAP expression domain.

The hormones responsible for the chromatin modifications surrounding the different rabbit hypersensitive sites remain unknown. Their identification would be a key step in elucidating the mechanism that controls WAP gene expression in the mammary gland. Various hormones are known to play a role

in chromatin remodelling. To date, a limited number of studies have been performed in the mammary-gland model. In MCF7, a human breast-cancer cell line, oestrogens were identified to play a role in the chromatin remodelling of the *pS2* gene upstream region [39]. In the $\beta$-lactogobulin gene, it has been suggested that PL may affect the chromatin structure surrounding the regulatory regions [13]. No PL has been detected so far in the rabbit. In the present study, we demonstrate that in rabbit primary mammary-gland culture cells, variations in the chromatin structure surrounding rabbit HSS0 and HSS2 depend on prolactin alone. In our model, the chromatin structure surrounding HSS0 and HSS2 was clearly independent of glucocorticoid treatment. Therefore under prolactin stimulation of the mammary-gland cells, the chromatin structure corresponds to that observed in the rabbit lactating mammary gland, where maximal expression of the WAP gene is detected. However, in rabbit primary mammary-gland culture cells stimulated by prolactin only, a low level of WAP mRNA was detected, as observed previously with the WAP–CAT construct in HC11 cells [25]. It is thus clear that an open chromatin structure is not sufficient for the full expression of the WAP gene. As full expression is observed under simultaneous prolactin and cortisol treatment, we conclude that both hormones are required to establish a chromatin structure that allows WAP gene expression and accumulation of WAP transcripts. Finally, differences exist between HSS0 and HSS2. In the rabbit mammary gland, whereas HSS2 disappears after weaning, HSS0 is still detected at this stage. HSS2 is the only hypersensitive site whose presence correlates with WAP gene expression, suggesting a key role for this in regulating this expression. Although both HSS0 and HSS2 are induced by prolactin, control of the chromatin structure surrounding these sites is not strictly identical.

Among cytokines, only interleukin-2 [40] and growth hormone [41] have been demonstrated to act in chromatin remodelling of the surrounding regulatory regions. Our results now provide evidence that prolactin is capable of inducing variations in the chromatin structure upstream of the WAP gene.

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