# A distal region, hypersensitive to DNase I, plays a key role in regulating rabbit whey acidic protein gene expression

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The aim of the present study was to identify the functional domains of the upstream region of the rabbit whey acidic protein (WAP) gene, which has been used with considerable efficacy to target the expression of several foreign genes to the mammary gland. We have shown that this region exhibits three sites hypersensitive to DNase I digestion in the lactating mammary gland, and that all three sites harbour elements which can bind to Stat5 *in vitro* in bandshift assays. However, not all hypersensitive regions are detected at all stages from pregnancy to weaning, and the level of activated Stat5 detected in the rabbit mammary gland is low except during lactation. We have studied the role of the distal site, which is only detected during lactation, in further detail. It is located within a 849 bp region that is required to induce a strong expression of the chloramphenicol acetyltransferase reporter gene in transfected mammary cells. Taken together, these results suggest that this region, centred around a Stat5-binding site and surrounded by a variable chromatin structure during the pregnancy–lactation cycle, may play a key role in regulating the expression of this gene *in vivo*. Furthermore, this distal region exhibits sequence similarity with a region located around 3 kb upstream of the mouse WAP gene. The existence of such a distal region in the mouse WAP gene may explain the differences in expression between 4.1 and 2.1 kb mouse WAP constructs.

Key words: mammary gland, milk, prolactin, Stat5.

## INTRODUCTION

The whey acidic protein (WAP) has been described in the milk of rodents [1], rabbits [2], camel [3], pig and Tammar wallaby [4], and its gene has been shown to be expressed specifically in the mammary gland. This expression has been shown to be regulated by lactogenic hormones, which are at extremely low levels outside the pregnancy–lactation period, and reach their highest levels during lactation. The upstream regions of the mouse WAP [5,6] and rabbit WAP [7,8] genes have thus been linked to heterologous genes and used to target the expression of those genes to the mammary gland of transgenic animals. The level of foreign gene expression in the mammary gland of lactating transgenic mice or rabbits varied, depending on both the WAP upstream region chosen and the gene that was expressed. An upstream region of 2.1 or 4.1 kb flanking the transcriptional start point (tsp) of the mouse WAP gene was able to control high-level expression of the transgene in the mammary gland during lactation, but was insufficient to allow properly regulated expression of the transgene during pregnancy [5]. Longer upstream regions of the rabbit WAP gene (from $-6.3$ kb or $-17.6$ kb down to position $+28$ relative to the tsp located at $+1$ [9]) enabled the attainment of levels that were reproducibly among the highest described [7,10]. Transgenes were mainly expressed in the mammary gland, and were correctly regulated during pregnancy. Results did not differ significantly between the longer and shorter rabbit WAP upstream regions, suggesting that the principal regulatory elements are located within the 6.3 kb region. However, the functional domains of this 6.3 kb rabbit WAP gene region have only been described poorly [9,11]. Such a small number of studies can be explained by the fact that WAP gene regulatory elements can only be studied clearly after the transfection of primary mammary cells, which are the only ones in which WAP gene expression is induced (as *in vivo*), in the presence of prolactin, and amplified in the presence of glucocorticoids. This induction of expression has not been detected in available mammary cell lines [12], and when those mammary cell lines are transfected with WAP constructs, glucocorticoids alone were capable of inducing transgene expression rather than amplifying prolactin activity as is the case *in vivo* [13].

Important regulatory elements of several genes have been located within DNA domains that are hypersensitive to mild DNase I treatments [14,15]. The presence of these hypersensitive sites, often detected around genes engaged in transcription, may vary during development or under hormone treatments, and reflect the accessibility of transcription factors to regulatory elements [16–18]. In order to describe the rabbit WAP upstream region more precisely, the aim of this study was to probe its chromatin structure in expressing and non-expressing tissue using DNase I hypersensitivity. In hypersensitive regions, the presence of regulatory elements was then sought by analysing the interactions between potential binding sites and transcription factors in bandshift assays. Finally, the functional role of these regions was confirmed after transfecting different chimaeric genes into primary mammary cells.

## EXPERIMENTAL

### Animals

White New Zealand rabbits at day 8 of pregnancy or at day 10 of lactation were killed in a slaughterhouse, in accordance with French Ministry of Agriculture guidelines (dated 19 April 1988). Lactating animals were killed less then 1 h after suckling by their

Abbreviations used: WAP, whey acidic protein; CAT, chloramphenicol acetyltransferase; HSS, DNase I-hypersensitive site; tsp, transcriptional start point; CHO, Chinese hamster ovary.

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young. In all cases, the mammary glands were dissected and stored at −20 °C until use.

### Construction of chimaeric genes

The −6359 and −3281 WAP/chloramphenicol acetyltransferase (CAT) constructs have already been described as pWAP/CAT-6300 and pWAP/CAT-3000 [11]. They were renamed relative to the tsp located at +1, after sequencing of the entire upstream region of the rabbit WAP gene [9]. The −5510 WAP/CAT construct was obtained after digestion with *Aat*II of the −6359 WAP/CAT construct and ligation of the *Aat*II site, which is located at −5510 upstream of the WAP gene, to the *Aat*II site in the flanking region of the pPolyIII vector [11].

### Cell cultures, transfections and hormone inductions

Chinese hamster ovary (CHO) $K_1$ cells were grown on 60 mm dishes. They were co-transfected with a prolactin-receptor-expressing plasmid (pE-$R_2$3, 3 $\mu$g), WAP/CAT constructs (2.5 $\mu$g) and a $\beta$-galactosidase-expressing plasmid (pCH110, 3 $\mu$g), and then subjected to prolactin treatment during the transient expression of transfected constructs, as described previously [19].

HC11 cells were grown and transfected with 8 $\mu$g of WAP/CAT DNA and 2 $\mu$g of pSVneo per 10 cm culture dish, as described previously [13]. G418-resistant cells were grown to confluency, pooled and split between several 60 mm dishes for hormone induction. Hormone treatments (insulin, 4 $\mu$g/ml; prolactin, 5 $\mu$g/ml; dexamethasone, $10^{-6}$ M) were performed as described previously [13]. For all constructs, equivalent amounts of DNA were transfected into cells, as monitored by the amount of transfected DNA detected/10 $\mu$g of total DNA (results not shown).

Primary organoids were prepared from the mammary glands of 14 day-old pregnant rabbits, plated on 60 mm dishes coated with 1 ml of rat-tail collagen gel and cultured for 4 days as described previously [11]. On the fourth day, cells were transfected and subjected to hormone treatment (prolactin, 1 $\mu$g/ml; dexamethasone, $10^{-6}$ M) for 72 h, as described previously [11]. The reproducibility of organoid preparations was checked by radioimmunoassay to detect the amount of $\beta$-casein secreted into the medium during the 72 h of hormone treatments [2].

### CAT assay

After hormone treatment, cells were recovered by centrifugation after scraping the plates into 0.5 ml of PBS or digesting the collagen gel with 570 units/ml collagenase in Hanks medium for 15 min at 37 °C. Cells were then resuspended in 100 $\mu$l of 10 mM Tris/HCl, pH 8.0, and lysed by three cycles of freezing and thawing. In mammary cell extracts, the protein content was measured and CAT activity evaluated for equal amounts of protein (2 $\mu$g), as described previously [11], or in CHO cells for equal amounts of $\beta$-galactosidase activity evaluated as described in [19].

### Isolation of nuclei and DNase I digestion

Nuclei were isolated from freshly excised tissue and DNase I digestion was performed as described by Whitelaw and Webster [20]. Genomic DNA was purified by three rounds 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 Pharmacia Biotech, Orsay, France). The specific activity of probes was in the order of $10^9$ c.p.m./$\mu$g. Radioactive signals were detected using STORM™ and analysed with ImageQuant™ software.

### DNase I footprint analysis of the upstream region of the rabbit WAP gene

DNase I footprint analysis was performed as described in [21], with the minor modifications which follow. Nuclear extracts were prepared using nuclei isolated from the mammary glands of rabbits at different stages (pregnancy, lactation and after weaning) or from rabbit liver [22]. The three different probes corresponding to the three DNase I-hypersensitive sites (HSSs), HSS2, HSS1 and HSS0, were prepared by amplifying the cloned WAP gene upstream region [7] using the following sets of primers: 5′-CACAGAGCTCTGGGGATGTG-3′ and 5′-GGTGGACGTCCCGTGCTGGC-3′; 5′-GCACCATAGTGCCCCGCACG-3′ and 5′-CGCAGGTGTCTGAGCCGTAG-3′; 5′-AGCCATCCTGCCCTGGGGTC-3′ and 5′-GGACTCTGTAAGCAAGAATG-3′, respectively. Prior to amplification, one of the two primers (1.5 pmol) was radiolabelled with [$\gamma$-$^{32}$P]ATP (ICN Pharmaceuticals, Orsay, France; 4000 Ci/mmol, 35 $\mu$Ci) and T4 kinase to prepare direct and reverse probes. Nuclear extracts (10 $\mu$g) were incubated with the different probes (10000 c.p.m.) for 30 min at room temperature and then digested for 1–3 min with 0.05–0.5 units of DNase I (Promega, Charbonnières, France) at room temperature.

### Electrophoretic mobility-shift assay

Probes end-labelled with [$\gamma$-$^{32}$P]ATP and T4 kinase (50000 c.p.m.) were incubated with 5 $\mu$g of nuclear extract for 30 min at room temperature, as described previously [23]. In some cases, nuclear extracts were pre-incubated for 30 min at room temperature with a 3–10-fold excess of unlabelled probe or with 1 $\mu$l of specific monoclonal antibodies against mouse Stat5a (Zymed, reference no. 13-3600; Upstate Biotechnology, reference no. 06 553) or Stat5b (Zymed, reference no. 13-5300; Upstate Biotechnology, reference no. 06 554) as described in the Figure legends. After the pre-incubation and incubation periods, samples were loaded on to a 5 % non-denaturing polyacrylamide gel in 12.5 mM Tris, pH 8.3/12.5 mM boric acid/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 with STORM™ and analysed with ImageQuant™ software.

### Northern blots

Total RNA were extracted from the mammary glands, analysed on 1 % agarose gel, transferred to Nytran and hybridized with an $\alpha$-$^{32}$P-labelled WAP or 18 S cDNA probe, as described previously [7]. Radioactive signals were detected using STORM™ and quantified with ImageQuant™ software.

## RESULTS

### Three HSSs are detected within the 6.3 kb upstream region of the WAP gene

Nuclei were isolated from the mammary gland of lactating rabbits and exposed or not to mild DNase I treatment. The DNA was then isolated, digested with several restriction enzymes and analysed on Southern blots with probes specific to the 5′ or 3′ ends of generated fragments. After digestion with *Bgl*II and hybridization with probe 3 (which is specific to the 5′ end of the

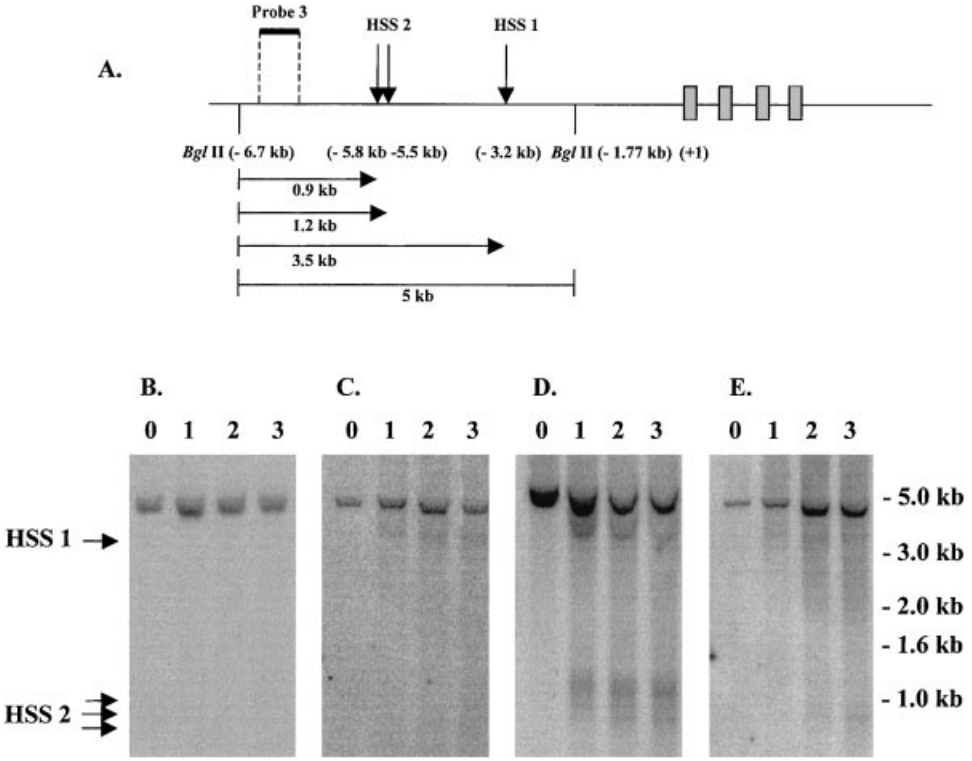


**Figure 1 Localization of the 5′ border of HSSs present in the upstream region of the WAP gene extracted from the mammary glands of lactating rabbits**

(**A**) Restriction map of the upstream region of the rabbit WAP gene. HSSs are indicated on the map by vertical arrows, fragments generated by DNase I are indicated by horizontal arrows. (**B**–**E**) Nuclei isolated from rabbit liver (**B**) or mammary gland (**C**–**E**) were incubated for 0, 3, 6 or 10 min (lanes 0–3 respectively) with DNase I (40 units). DNA was then isolated and digested with *Bgl*II, and the fragments generated were separated by electrophoresis on 1% agarose gels before being transferred to Nytran N$^{TM}$ and hybridized with probe 3. Fragment size was determined by comparison with those of the kb ladder. (**C**–**E**) Mammary glands were isolated from rabbits on day 8 of pregnancy (**C**), on day 12 of lactation (**D**) or 1 month after weaning (**E**). These Figures are representative of the very similar results which were obtained for nuclei isolated from three different rabbits for each stage or tissue.

*Bgl*II fragment generated from the WAP upstream region; Figure 1A), the principal signal was observed as expected for a 5 kb fragment (Figure 1D, lane 0). Mild DNase I treatment induced the appearance of several bands (Figure 1D, lane 1), which were not detected in the liver (Figure 1B, lanes 0 and 1). A sharp band corresponds to a 3.5 kb fragment and several more diffuse bands correspond to 0.9–1.2 kb fragments. All these fragments were due to the digestion of regions hypersensitive to DNase I. These regions were mapped not only with reference to the 5′ end (Figure 1A) of the restriction fragments encompassing them but also with reference to the 3′ ends (Figure 2 and results not shown). As expected, the signal observed for HSS1 was then weaker than observed in Figure 1(D). This could be explained by the presence of another hypersensitive site located between the probe and HSS1 (see below). Both HSS2 and HSS1 were analysed further for evidence of multiple, very close, hypersensitive sites. HSS1 was estimated to cover from $-3.2$ to $-2.5$ kb relative to the tsp (Figures 1A and 2A), whereas HSS2 extended from $-5.8$ to $-5.4$ kb (Figure 1A and results not shown). Two other hypersensitive regions were also detected. One is located at approx. $-500$ bp from the tsp (HSS0, Figure 2D, lane 1), in the proximal upstream region of the gene, while the other is located at around $+500$ bp in the coding region of the gene. Although the signal corresponding to this last site was very strong in one of the animals studied, it was not observed in other animals and was not characterized further. None of these HSSs were detected in the liver (Figures 1B and 2B).

### Footprint analysis of HSS2 highlighted different patterns in the liver and mammary gland

To further characterize the HSS, *in vitro* footprint analyses were performed using nuclear extracts from the liver and mammary gland of pregnant, lactating and post-weaning rabbits. End-labelled probes covering each hypersensitive site were used to determine more precisely the positions of DNA regions interacting with transcription factors *in vitro*. Only probes covering HSS2 clearly exhibited different patterns for liver and lactating mammary gland extracts (Figure 3). However, no differences were observed between the different stages of mammary gland development.

The absence of clear footprint patterns was consistent with low-affinity protein–DNA interactions. Whereas these *in vitro* data are interesting, they do not enable the identification of transcription factors interacting *in vivo* with those regions.

### The three HSSs encompass three short sequences which bind Stat5 *in vitro*

The three HSSs described above are located within a region extending between $-6359$ bp from the tsp of the rabbit WAP gene and $+2336$ bp. The sequence of this region has been described and scanned for transcription-factor consensus binding sites using TFSEARCH version 1.3 [24]. Within this 8695 nt sequence, several sequences having some similarity with con-

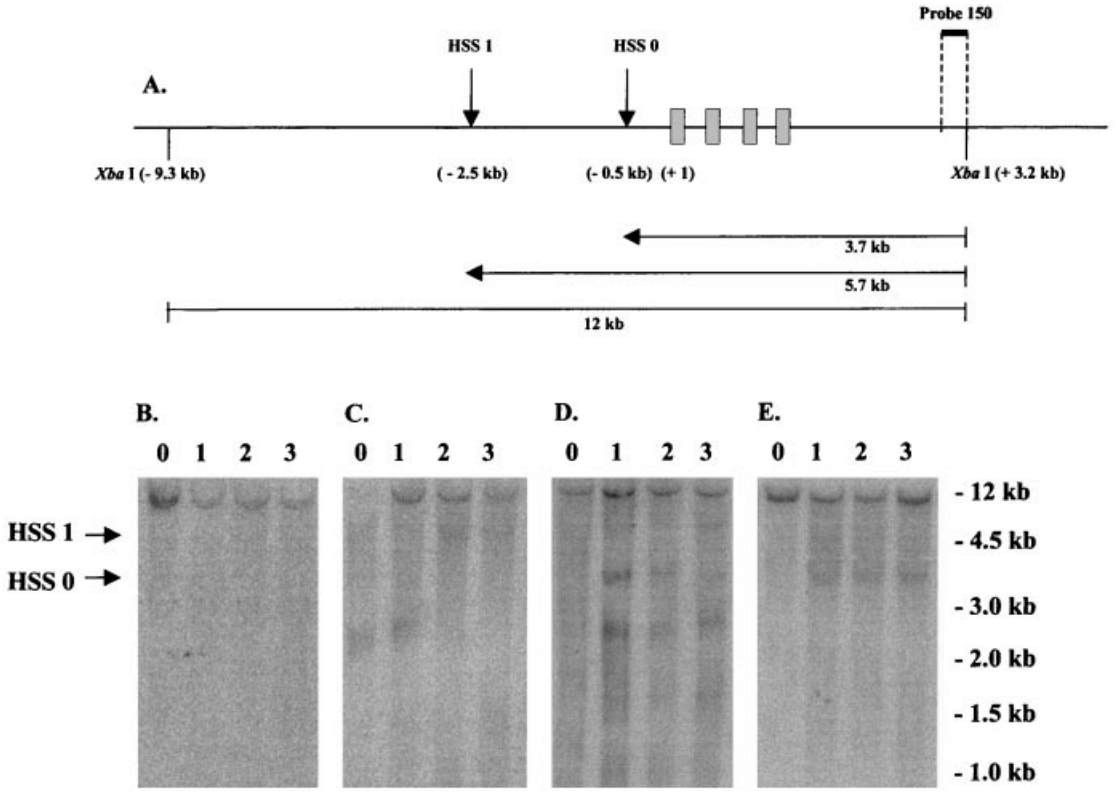


**Figure 2 Localization of the 3′ border of HSS1 and identification of HSS0 present in the upstream region of the WAP gene extracted from the mammary gland of lactating rabbits**

(**A**) Restriction map of the rabbit WAP gene. HSSs are indicated on the map by vertical arrows, fragments generated by DNase I are indicated by horizontal arrows. (**B**–**E**) Nuclei isolated from rabbit liver (**B**) or mammary gland (**C**–**E**) were incubated for 0, 3, 6 or 10 min (lanes 0–3 respectively) with DNase I (40 units). DNA was then isolated and digested with *Xba*I, and the fragments generated were separated by electrophoresis on 1% agarose gels before being transferred to Nytran N$^{TM}$ and hybridized with probe 150. Fragment size was determined by comparison with those of the kb ladder. (**C**–**E**) Mammary glands were isolated from rabbits on day 8 of pregnancy (**C**), on day 12 of lactation (**D**) or 1 month after weaning (**E**). These Figures are representative of the very similar results which were obtained for nuclei isolated from three different rabbits for each stage or tissue.

sensus for CCAAT-enhancer-binding protein (C/EBP)-, c-ets ('cellular homologue of viral E-26-specific sequence')- and NF-1 (nuclear factor 1)-binding sites were observed all along the sequence, whereas three short sequences, −5604 to −5588, −3032 to −3013 and −569 to −553 relative to the tsp of the WAP gene, exhibited strong similarity with the Stat5 consensus sequence (Table 1). We therefore focused on these three potential Stat5-binding sites, given their strong similarity with the consensus sequence and the involvement of this transcription factor in milk-protein gene expression. Unlike C/EBP, c-ets and NF-1, these three sequences are located within the three hypersensitive regions identified above. Double-strand oligonucleotides corresponding to these sequences (probes 2, 1 and 0; see Table 1) were synthesized and radiolabelled with $^{32}$P. When incubated in the presence of mammary gland nuclear extracts isolated from lactating rabbits, the three probes were able to bind proteins and form complexes (Figure 4, panels 0–2, lanes −), with the same electrophoretic mobility as the Stat5-specific complex formed with an Stm probe designed from the proximal upstream region of the ovine $\beta$-lactoglobulin gene [22] (Figure 4, panel S). The intensity of signals observed with the different probes varied, but the variations were not reproducible; they were mainly dependent on the preparation of probes and not considered to be significant.

Two isoforms of Stat5, Stat5a and Stat5b, have been described in the mouse and human, and monoclonal antibodies specific to each form are available. These antibodies were used to determine which isoforms of Stat5 were present in DNA-binding complexes. Complexes formed in the presence of probes 0, 1, 2 or S could be partially shifted with Stat5a- or Stat5b-specific antibodies (Figure 4, lanes a or b), and were totally shifted when both antibodies were present simultaneously (Figure 4, lanes a,b). Supershift signals were stronger than shift signals, as has already been observed using certain Stat5a antibodies [25]. All complexes formed with probes 0, 1, 2 or S thus included Stat5a and/or Stat5b.

No complexes were observed when the probes introduced into the assay carried mutations on the two nucleotides known to be important to Stat5 binding [22,26] (results not shown). We therefore conclude that sequences 0, 1 and 2, which are localized in open chromatin structures in the rabbit mammary gland during lactation, are potential targets for activated Stat5.

### The level of Stat5 isoforms binding to DNA reaches a maximum during lactation

The results described above were observed in rabbit mammary glands during lactation when the organ was well developed and when activated Stat5 was present in large quantities in the nucleus. However, Stat5 activity has not yet been described in mammary gland extracts at early stages of pregnancy or after weaning in the rabbit. On day 7 or 8 of pregnancy, in rabbits, the mammary gland is still poorly developed and WAP mRNA is

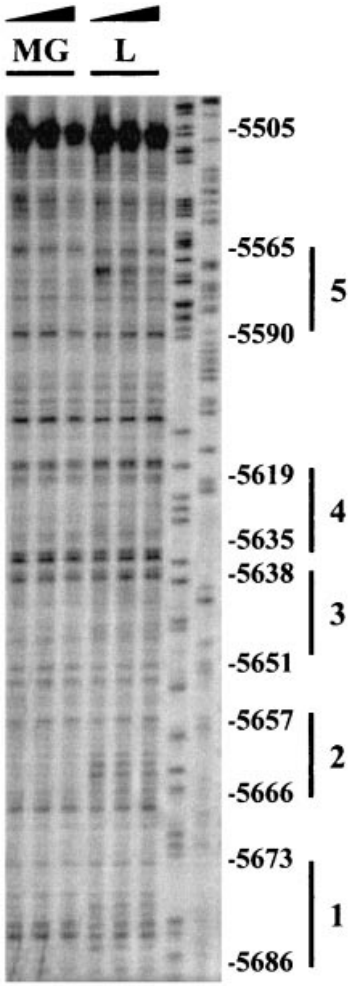


**Figure 3 DNase I footprint analysis of the HSS2 region (−5751 to −5505)**

Experiments were performed using nuclear extracts from lactating mammary gland (MG) or liver (L). On the right, G/A sequencing tracks, used as molecular-mass markers, are shown. The bars on the right indicate patterns of bands which differ in mammary gland and liver extracts (1–5). The position of these regions is given relative to the tsp.

**Table 1 Sequences of the oligonucleotides employed**

Double-stranded oligonucleotides used in bandshift assays are listed below. The ovine $\beta$-lactoglobulin Stm probe was used as a standard [22]. The localizations of other probes relative to the tsp of the rabbit WAP gene are shown in brackets. Their sequences are compared with the Stat5 consensus sequence [26]; bases matching the Stat5 consensus sequence are shown in bold. Bases which have been changed in the sequence of mutated probes are underlined.

| Type of probe | Sequence |
|---|---|
| Stat5 consensus sequence | 5′-**ANTTCTTGGAA**-3′ |
| Stm probe | 5′-GA**TTC**CG**GGAA**CCGCG-3′ |
| | 3′-T**AAG**GC**CCTT**GGCGCA-5′ |
| Probe 0 (−569 to −553) | 5′-CC**TTC**CGA**GAA**GAGACC-3′ |
| | 3′-GG**AAG**GCT**CTT**CTCTGG-5′ |
| Probe 1 (−3032 to −3013) | 5′-GGC**TTCT**GA**GAA**TCTCCA-3′ |
| | 3′-CG**AAGA**CT**CTT**AGCGGTC-5′ |
| Probe 1m (mutated probe 1) | 5′-GC**TTCT**GCT**AA**TCTCCA-3′ |
| | 3′-CG**AAGA**CGA**TT**AGAGGT-5′ |
| Probe 2 (−5604 to − 5588) | 5′-CC**TTC**C**TGGAA**CCGTC-3′ |
| | 3′-G**AAG**G**ACCTT**GGCAGA-5′ |
| Probe 2m (mutated probe 2) | 5′-CC**TTC**CTCT**AA**CCGTCT-3′ |
| | 3′-GG**AAG**GAGA**TT**GGCAGA-5′ |

only observed at very low levels (Figure 5A, bar P). One month after weaning, the mammary glands, considered as a whole, were much less well developed than during lactation, but were still larger than on day 8 of pregnancy. The lobuloalveolar development observed after whole-mount staining was very similar to that observed on day 8 of pregnancy (results not shown). In two out of the three rabbits studied, only low levels of WAP mRNA were detected (Figure 5A, bar W).

When nuclear extracts were prepared from the mammary glands of 8 day pregnant rabbits (Figure 5B, P) and from rabbits 1 month after weaning (Figure 5B, W) similar results were observed in both cases. The amounts of complexes were much lower than those observed in two out of three rabbits studied during lactation (Figure 5B, L) and slightly lower than the level of Stat5 in the third lactating rabbit. The latter appeared to differ from the other two and was not representative of the lactating stage for two reasons: first, she had only seven pups instead of ten, and secondly, she had not been bred with the others and may have been sensitive to seasonal effects, a well-recognized phenomenon in rabbit breeding.

A low level of activated Stat5 is thus already detected at an early stage of pregnancy and is still detected 1 month after weaning. Since activated transcription factors were detected at both stages, it was important to study the presence of hypersensitive regions at those stages of mammary gland development in order to evaluate the potential binding of these transcription factors *in vivo*.

### The signals detected for HSS2 vary during mammary gland development

On day 7 or 8 of pregnancy, when WAP mRNA accumulation has not yet been induced by lactogenic hormones, HSS1 was clearly present, HSS2 was detected as a very faint band in only one of the two rabbits studied (Figure 1C) and HSS0 was not observed (Figure 2C).

One month after weaning, when only low levels of WAP mRNA were detected (Figure 5A, bar W), HSS1 was detected as a faint signal, HSS2 was not detected (Figure 1E) and HSS0 was clearly present (Figure 2E). When no HSSs were observed or only detected as a faint signal, DNA was checked on ethidium bromide-stained agarose gels before DNase I treatment. Laddering or smearing was never detected, even 1 month after weaning, showing that the absence of HSSs was not due to apoptosis or DNA degradation.

We therefore concluded that, among the three HSSs characteristic of the mammary gland, HSS1 is detected at all stages of mammary gland development but as a more or less pronounced signal, depending on the developmental stage, and HSS0 is not detected on day 8 of pregnancy but is present during lactation and remains 1 month after weaning. In contrast, HSS2, which is present on day 8 of pregnancy, disappears with weaning. Since HSS2 is the only site whose presence is associated with WAP mRNA expression, it was important to assay its functional role.

### The more distal HSS2 corresponds to a functional region in transfection assays

HSS0, HSS1 and HSS2 are included within 6359 nt of the upstream region of the WAP gene. This upstream region, down to position 27 of the 5′ untranslated region of the WAP gene, had

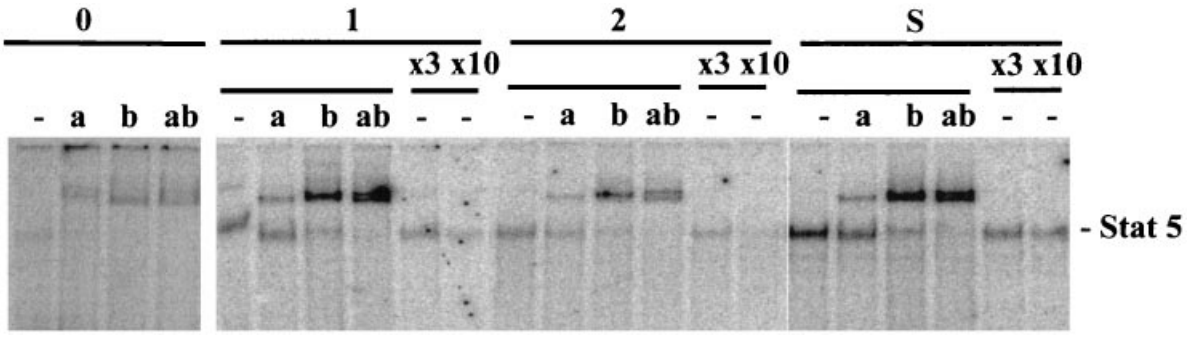


**Figure 4 Bandshift analysis of rabbit mammary gland nuclear extracts**

Probes 0, 1, 2 or S were incubated with nuclear extracts of lactating mammary glands (lanes —), in the presence of antibodies directed against mouse Stat5a (lanes a), mouse Stat5b (lanes b) or both Stat5a and Stat5b (lanes ab). Complexes were less abundant when the nuclear extracts were preincubated with a 3- or 10-fold excess of unlabelled probe ( $\times 3$ and $\times 10$). Similar results were observed using nuclear extracts isolated from two other lactating rabbits.

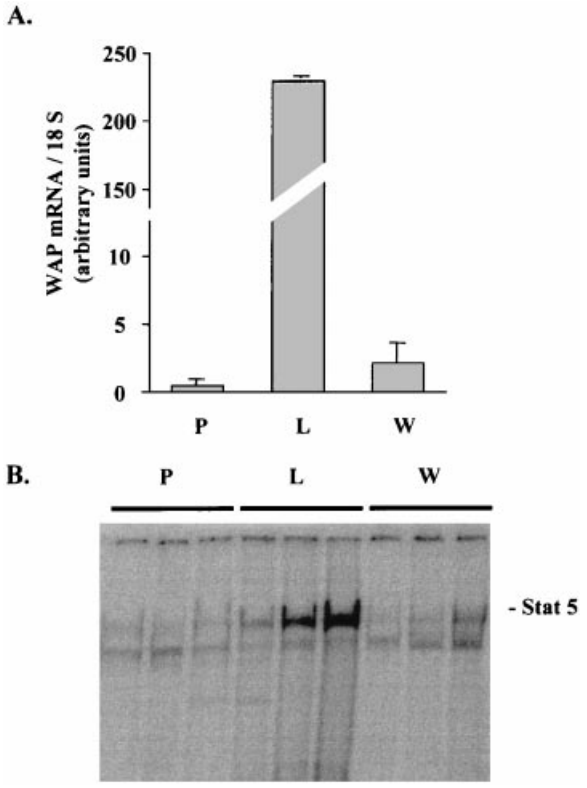


**Figure 5 Differences in WAP mRNA accumulation levels and Stat5-binding activities in the rabbit mammary gland during pregnancy, lactation and weaning**

(**A**) Accumulation of WAP mRNA in the mammary gland on day 8 of pregnancy (P), on day 12 of lactation (L) or 1 month after weaning (W). (**B**) Comparison of Stat5-binding activity in nuclear extracts from mammary gland on day 8 of pregnancy (P), on day 12 of lactation (L) or 1 month after weaning (W); three animals/stage. Nuclear extracts were incubated with probe S.

previously been linked to the bacterial CAT gene (WAP/CAT construct) and transfected into rabbit primary mammary cells. Two important functional regions had been located after transfection of this and a shorter construct. One region was identified between $-6359$ and $-3281$ bp and the other between $-3281$ and $-1806$ bp [11]. Each of these regions thus covers the HSSs described above, named HSS2 and HSS1 respectively. In order to locate more precisely the functional regulatory elements in the $-6359$ to $-3281$ bp region, another construct composed of 5510 bp of the upstream region of the rabbit WAP gene was recently built in a similar manner, and the transcriptional activity of constructs $-6359$ WAP/CAT, $-5510$ WAP/CAT and $-3281$ WAP/CAT assayed in three different cellular environments.

The three constructs were first co-transfected into CHO cells with a rabbit prolactin-receptor-expressing plasmid, and CAT activity was measured during transient expression of the chimaeric gene. No induction of CAT activity by lactogenic hormones was observed (Figure 6A), even if during the same experiment CAT activity could be induced from the $\beta$-lactoglobulin promoter, as described previously [19]. This WAP upstream region is thus not functional in non-mammary cells, even if the prolactin-receptor transduction pathway is functional in these cells to activate another milk-protein gene.

The three chimaeric genes were therefore also transfected into HC11 mouse mammary cells. Pools of transfected cells were amplified and CAT activity measured in cells treated or not with hormones during stable expression of the reporter gene. Strong CAT activity could be induced by dexamethasone alone when a construct carrying the 6359 bp of the upstream region of the rabbit WAP gene was transfected (Figure 6B). This CAT activity was only slightly higher in the presence of prolactin. This regulation of transfected gene expression by glucocorticoids in HC11 cells has already been described [13]. In our studies, the induction of CAT activity by lactogenic hormones was reduced when only a 5510 bp fragment was used. A further deletion of the construct to $-3281$ had no further effect on CAT activity, which was still inducible by lactogenic hormones. An important regulatory region is therefore located between $-6359$ and $-5510$ bp from the tsp and corresponds to the HSS2 region. However, because the hormonal regulation of CAT activity differs from that of milk-protein gene expression *in vivo*, it was necessary to further assess these results using another cell model.

The activity of constructs was therefore assayed in rabbit primary cells, in which previous experiments had shown that the hormonal regulation of transfected genes was correct [11]. The chimaeric genes were transfected into those cells and CAT activity was measured after treating the cells for 72 h, with or without lactogenic hormones, during transient expression of the

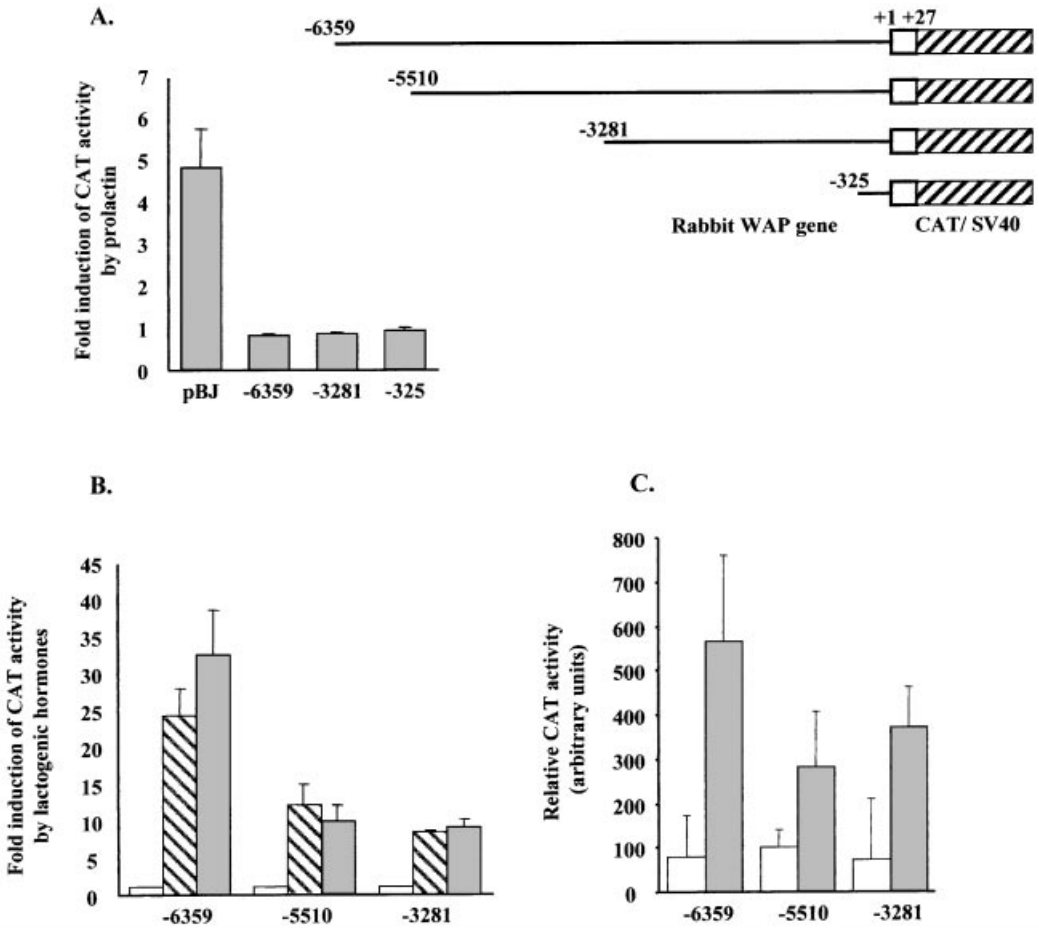


**Figure 6 Induction of CAT activity by lactogenic hormones in mammary cells transfected with WAP/CAT constructs**

These constructs contained the 6359 bp, 5510 bp or 3281 bp upstream region of the rabbit WAP gene to position +27 relative to the tsp, ligated to a CAT expression vector which included the intron and polyadenylation signal of simian virus 40 (SV40, striped box). (**A**) CAT activity in CHO cells co-transfected with WAP/CAT constructs and a rabbit prolactin-receptor-expressing plasmid. CAT activity was measured after prolactin stimulation during transient expression of the chimaeric gene. The $\beta$-lactoglobulin promoter (pBJ) was used as a positive control. Values are means ± S.E.M. from values observed in three independent dishes. (**B**) Lactogenic hormones induced stable CAT activity in transfected HC11 mouse mammary cells. CAT activity was evaluated in pools of transfected HC11 cells in the absence or presence of insulin (white boxes), insulin and dexamethasone (striped boxes), or insulin, dexamethasone and prolactin (grey boxes). Values are means ± S.E.M. from four independent pools of transfected cells. (**C**) Lactogenic hormones induced transient CAT activity in transfected rabbit primary mammary cells. CAT activity was evaluated in transfected cells treated with insulin (white boxes), or insulin, dexamethasone and prolactin (grey boxes). Values are means ± S.E.M. from four independent dishes. This result is representative of three independent experiments.

reporter gene. CAT activity could clearly be induced by lactogenic hormones when a construct carrying the WAP 6359 bp upstream region was transfected (Figure 6C), thus confirming previous results [11]. The induction of CAT activity by lactogenic hormones was reduced when only a 5510 bp fragment was used. A further deletion of the construct to −3281 had no further effect on CAT activity, which was still inducible by hormones, as described previously [11].

Functional regulatory elements are thus located between −6359 and −5510 bp, within the HSS2 region, and are necessary for lactogenic hormones to induce strong and transient CAT expression in primary mammary cells. However, these elements were not able to confer prolactin regulation on the thymidine kinase promoter when such constructs were transfected into rabbit primary mammary cells (results not shown). This result was not surprising since the three Stat5 elements in the $\beta$-lactoglobulin promoter are also required simultaneously to obtain strong expression in both HC11 and transgenic mice [22]. It is thus likely that the Stat5 elements present in the three HS regions of the WAP gene are alone insufficient to control gene expression, but belong to a composite regulatory region spread over large regions around the rabbit WAP gene.

## DISCUSSION

The results we describe here demonstrate the existence of at least three important regulatory regions upstream from the rabbit WAP gene. The importance of these regions, which have recently been sequenced [9], is confirmed by comparing the results reported above with the data available on rat and mouse WAP genes. The sequence underlying the more proximal HSS0 site in the rabbit may be aligned with the rat WAP gene sequence from −788 to −717. This rat WAP gene sequence, in turn, overlaps with the more distal hypersensitive site described for the rat gene from −853 to −729 [17], which like rabbit HSS0 corresponds to a Stat5-binding site. The role of this rat hypersensitive site has been emphasized by the fact that this Stat5-binding site, together with an NF-1 regulatory element located close by, is required to

control high levels of transgene expression in the mammary gland of lactating mice [15]. Its importance is also suggested by the fact that the appearance of this rat HSS can be induced *in vitro* by glucocorticoid treatments [17]. Interestingly, the region corresponding to rabbit HSS0 may also be aligned with the upstream sequence of the mouse WAP gene from −832 to −533. This mouse sequence is flanked by glucocorticoid-receptor-binding sites from −231 down to the tsp [27] which are not conserved in the rabbit. It also flanks an ets-1 (a transcription factor encoded by the c-ets-1 gene) site located around −110, which is conserved between rabbit, mouse and rat [28,29], and which has been shown to be functional both *in vivo* in transgenic mice [28] and *in vitro* [29]. By comparing our results with those obtained for rat and mouse WAP genes we therefore highlight a potential role for the rabbit proximal region. It would have been interesting to analyse this rabbit HSS0 region and map its regulatory elements by functional assays. However, since the signals observed in transfection assays, with short proximal regions only, are very weak in both the rabbit (results not shown) and mouse [13] WAP genes and since this region of the WAP gene has already been largely described in other species, the rabbit WAP gene proximal region has not been characterized further. The work described herewith focused on describing a more upstream region where we located two functional regulatory subregions that have not yet described in any other WAP gene.

Each of these subregions contains an HSS (HSS1 and HSS2, respectively) and a Stat5-binding element, and corresponds to functional regions in transfection assays, as shown above. The sequences underlying HSS1 and HSS2 could not be aligned with the rat WAP gene, which has only been described up to −1191 bp (GenBank accession no. RNWAP1) from the tsp. However, they could be aligned with two regions of the mouse WAP gene (GenBank accession no. MMWAPX1), from −1281 to −1407 bp and from −1866 to −3066 bp, respectively. In each case, these mouse regions included not only the conserved sequence of a Stat5-binding element but also their flanking regions, suggesting that in both distal and proximal regulatory regions a complex set of regulatory elements are involved in WAP gene transcription (see the discussion above).

Our results therefore map important regulatory regions in the rabbit WAP gene and allow prediction of the location of important regulatory regions in the mouse WAP gene. They show that regulatory regions extending from −6359 to the tsp in the rabbit may be closer to each other in the mouse WAP gene, extending only from −3.1 kb to the tsp. The existence of a regulatory region between 3.1 and 2.1 kb in the mouse WAP gene would explain the differences in the expression of 4.1 and 2.1 kb mouse WAP constructs.

It would have been very interesting to study the effect of single, double and triple mutations/deletions of Stat5 sites on the activity of −6359/+29 CAT reporter constructs, in the absence or presence of lactogenic hormones. However, we decided not to investigate this further because in another milk-protein gene, the ovine $\beta$-lactoglobulin promoter, it has been shown that all three Stat5 sites located in the proximal region contribute towards maximum expression of the $\beta$-lactoglobulin gene in transgenic mice [22]. Furthermore, it has been demonstrated that a single mutation of a Stat5 site in the rat WAP gene proximal region lowers WAP expression levels to 10% of that of the wild type [15]. It was necessary for these experiments to be carried out using transgenesis, because the level of gene expression observed after mutation was too low to be observed in the only available functional assay: primary mammary cells.

The chromatin structure surrounding the ovine $\beta$-lactoglobulin gene has also been described [30], showing the existence of two major HSSs in the proximal upstream region of the gene. A major HSS has been detected (HSS III) around −300 bp from the cap site. It corresponds to three Stat5-binding sites and when it is present in a 406 bp fragment this short promoter is sufficient to target the expression of a foreign gene to the mammary gland of transgenic mice during lactation. A weaker site is observed at −1800 bp from the cap site, but its regulatory elements have not been described. These two sites appear during late pregnancy (day 100, whereas the length of ovine pregnancy is 120 days) and are clearly detected during lactation. This opening of the chromatin structure was assumed to be due to an increase in placental lactogen hormone levels [30].

In the rabbit WAP gene, hypersensitive sites such as HSS1 are already detected at day 7 or 8 of pregnancy (length of pregnancy in the rabbit is 28 days). In this species, an open chromatin structure, as probed by DNase I hypersensitivity, is thus detected much earlier during pregnancy than in sheep. At that stage, transcription of the gene is still so weak that it had not been detected previously [31] and can only now be detected using more sensitive techniques (Figure 5A). This low level of WAP gene transcription is associated with the presence of activated Stat5 which is also detected at low levels. However, opening of the chromatin structure at HSS1 cannot be due to placental lactogen hormone since this hormone is supposed not to exist in this species. This variation in chromatin structure may happen at the very beginning of pregnancy and be due to the slight increase in prolactin levels which is observed at that time in the rabbit [32]. Alternatively, this opening may occur when the mammary gland develops before pregnancy during ovarian cycles, at puberty or even during mammary gland foetal development. A very early variation in chromatin structure before or at puberty could then explain why expression of milk protein can be induced in the virgin rabbit by the injection of prolactin alone [33], and WAP mRNA can be detected by reverse transcriptase PCR in the mammary gland before pregnancy [8]. However, this WAP mRNA signal is very weak and cannot be compared with the variation in WAP gene transcription observed in cyclic mice, since no ovarian cycles are observed in the rabbit.

Unlike HSS1, which is detected at all stages of development, HSS2 disappears between two cycles of lactation, when WAP mRNA accumulation returns to basal levels. The chromatin structure surrounding the WAP gene then loses some characteristics of the lactation stage. The appearance of HSS2, which is always detected when WAP mRNA are expressed, must therefore be induced anew at each pregnancy. The inducing hormones may be prolactin, glucocorticoids or progesterone, levels of which rise during the early stages of pregnancy and which have been shown to be capable of modifying the chromatin structure; glucocorticoids would be active in the mammary gland [17], prolactin and progesterone in other tissues.

The results we describe therefore suggest important roles for the region underlying HSS2. However, they also show that the regions underlying the constitutive HSS1 are sufficient to obtain a weak regulated expression of a reporter gene in transfection assays, indicating that the distal and proximal regions are most likely to interact *in vivo* to form powerful regulatory regions of the rabbit WAP gene. The presence of rabbit WAP distal regions in the constructs used for transgenesis experiments explains the high level of expression observed [7,10]. However, although transgene expression was always strong, it was never copy-dependent, indicating that the transgene was not an independent transcription unit. Other regulatory elements (such as insulators) are therefore located elsewhere around the rabbit WAP gene, perhaps in the large region which has now been cloned into a BAC vector [9].

We thank J. Djiane for his permanent interest, encouragement and positive comments during the course of this work, B. Whitelaw for his support and critical reading of the manuscript, G. Jolivet, N. Daniel, I. Gourdou, M. Lemnaouar and G. de Bruyn for their help in bandshift and footprint analyses and cell cultures, and V. Hawken for correcting the English. The work was supported by the Institut National de la Recherche Agronomique. B. M. was supported by a fellowship from the Ministère de la Recherche et de la Technologie, France.

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Received 23 July 2001; accepted 16 August 2001