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The Nuclear Localization of WAP and CSN Genes is Modified by Lactogenic Hormones in HC11 Cells

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ABSTRACT

Whey acidic protein (*WAP*) and casein (*CSN*) genes are among the most highly expressed milk protein genes in the mammary gland of the lactating mouse. Their tissue-specific regulation depends on the activation and recruitment of transcription factors, and chromatin modifications in response to hormonal stimulation. We have investigated if another mechanism, such as specific positioning of the genes in the nucleus, could be involved in their functional regulation. Fluorescent in situ hybridization was used to study the nuclear localization of *WAP* and *CSN* genes in mouse mammary epithelial cells (HC11) cultured in the absence and presence of lactogenic hormones. Automatic 3D image processing and analysis tools were developed to score gene positions. In the absence of lactogenic hormones, both genes are distributed non-uniformly within the nucleus: the CSN locus was located close to the nuclear periphery and the *WAP* gene tended to be central. Stimulation by lactogenic hormones induced a statistically significant change to their distance from the periphery, which has been described as a repressive compartment. The detection of genes in combination with the corresponding chromosome-specific probe revealed that the CSN locus is relocated outside its chromosome territory following hormonal stimulation, whereas the *WAP* gene, which is already sited more frequently outside its chromosome territory in the absence of hormones, is not affected. We conclude that milk protein genes are subject to nuclear repositioning when activated, in agreement with a role for nuclear architecture in gene regulation, but that they behave differently as a function of their chromosomal context. J. Cell. Biochem. 105: 262–270, 2008.

KEY WORDS: WHEY ACIDIC PROTEIN; CASEIN; PROLACTIN; CHROMOSOME TERRITORIES; 3D-FISH

The mouse mammary gland is a complex tissue which develops under the influence of systemic hormones during puberty, pregnancy, and early lactation. These developmental stages are critical to transforming the rudimentary ducts that are established during fetal life into a fully functional organ which is able to secrete high levels of milk proteins during late lactation [Rudolph et al., 2003; Brisken and Rajaram, 2006]. The Whey Acidic Protein (*WAP*) and casein (*CSN*) genes are among the most highly expressed milk protein genes in the mammary gland of lactating mice [Rijnkels et al., 1997; Rudolph et al., 2003]. During the pregnancy/lactation cycle, lactogenic hormones (mainly prolactin and glucocorticoids) induce the activation of several transcription factors which interact with the regulatory regions of milk protein genes to induce their expression [Rosen et al., 1999]. However, the presence of these transcription factors is not sufficient to explain mammary gland-specific expression, as they can also be detected in several other tissues [Ollivier-Bousquet and Devinoy, 2005].

The WAP gene is mapped close to the centromere on the gene-rich chromosome MMU11. It is located between two genes (*TBRG4* and *RAMP3*) which are expressed in several tissues [McLatchie et al., 1998; Rival-Gervier et al., 2003] that are not regulated by lactogenic hormones. Three functional Stat5-binding sites have been described in the upstream region of the *WAP* gene [Li and Rosen, 1995; Millot et al., 2003]. During lactation, prolactin activates Stat5 [Liu et al., 1996] which interacts with its three target sequences. In the rabbit, this interaction is regulated by variations in the chromatin structure which adopts a fully open conformation in the mammary gland, thus enabling the accessibility of these target sequences [Millot et al., 2003].

The mouse *CSN* locus consists of five genes (*CSN1s1*, *CSN2*, *CSN1s2a*, *CSN1s2b*, and *CSN3*) clustered in a 260-kb genomic DNA

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region on chromosome MMU5 [Rijnkels et al., 1997]. Their expression is regulated in a coordinated fashion during mammary gland development. Although several conserved binding sites for the same set of transcription factors have been identified in their proximal 5′-flanking regions, it has been suggested that there is a dominant control element which coordinates their expression [Rijnkels et al., 1997; Kolb, 2002].

While the activation and recruitment of transcription factors and chromatin modifications have been described as processes that are of importance to the regulation of milk protein gene transcription and mammary gland-specific expression [Schmitt-Ney et al., 1991; Millot et al., 2003; Kabotyanski et al., 2006], other mechanisms (not yet described in the mammary gland) may also play an important role in gene regulation. These may involve the positioning of genes within the nucleus, this having been related to their functional regulation. Nuclear periphery and centromeric heterochromatin constitute transcriptional repressive compartments where some silenced genes are sequestered [Brown et al., 1997; Francastel et al., 2001; Kosak et al., 2002; Zink et al., 2004]. Moreover, the positioning of genes relative to their chromosome territories (CT) is correlated with their expression [Volpi et al., 2000; Williams et al., 2002; Chambeyron and Bickmore, 2004], with some actively transcribed genes looping out of their CT to colocalize both *in cis* and *in trans* in transcription factories. They can also associate to ensure expression or co-regulation with regulatory regions or genes [Fraser and Bickmore, 2007].

In order to investigate whether the position of milk protein genes is related to their functional regulation, we have used a mouse mammary epithelial cell line (HC11) in which milk protein gene expression can be induced by lactogenic hormones [Doppler et al., 1989]. Previous studies in HC11 cells using the technique of nuclear halos—where the extraction of histones and other soluble proteins using hypertonic conditions causes the distension of DNA loops that do not strongly interact with the nuclear structure [Gerdes et al., 1994]—determined a correlation between the attachment of the chromatin loop surrounding a transfected *CSN* gene to the nuclear matrix and its level of expression [Poussin et al., 2005]. Similar results were recently obtained by our group, indicating that endogenous *WAP* and *CSN* genes are more frequently retained within the nucleus in HC11 cells cultured in the presence of lactogenic hormones [Montazer-Torbati et al., 2008].

Our results constitute the first demonstration of the nuclear organization of milk protein genes in mouse mammary epithelial cells. In HC11 cells cultured in the absence of hormones, these milk protein genes display a non-random positioning with respect to the nuclear periphery. After hormonal treatment and gene induction, significant changes can be seen to the localization of genes relative to the nuclear periphery or CT.

MATERIALS AND METHODS

CELL CULTURE

HC11 mouse mammary epithelial cells and 3T6-Swiss mouse fibroblast cells from ATCC (LGC Promochem) have a duplicated genome [Todaro and Green, 1963; Poussin et al., 2005, respectively]. HC11 cells were grown in RPMI-1640 supplemented with 10% fetal calf serum (HyClone, Perbio), 10 ng/ml EGF and 4 μg/ml insulin. 3T6 cells were cultured in Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose and 10% fetal calf serum. Cells were cultured on plastic Petri dishes at 37°C in a 5% CO_2 atmosphere. To promote differentiation, confluent cells were maintained for 5 days in the growth medium containing 10% fetal calf serum, 4 μg/ml insulin and 10^{-6} M dexamethasone. Milk protein gene expression was then induced for 2 days in the same medium plus 5 μg/ml prolactin.

TOTAL RNA ISOLATION AND RT-PCR

Total RNA was isolated from cells using Trizol (Invitrogen, Life Technologies). Two micrograms of total RNA was reverse-transcribed with 250 ng oligo(dT) (Sigma), 400 U Superscript II (Invitrogen) in the presence of 20 U RNAsin (Promega), according to the manufacturer's protocols. As a control, an equivalent amount of RNA was incubated without Superscript. The primers used to detect the cDNAs of interest were: WAP, 5′-CCTGACACCGGTACCATGCG-3′/5′-AGCATATTGAAAGCATTATGTTC-3′; CSN2, 5′-GATGTGCTC-CAGGCTAAAGT-3′/5′-ACGGAATGTTGTGGAGTGGC-3′; RAMP3, 5′-TGGGCTGCTACTGGCCCAACCC-3′/5′-CTGCGCCACACCACCAG-GCCAG-3′; TBRG4, 5′-CTGGGTTCTGACCATAGCCTGCACC-3′/5′-TCTCCAGGTGCATTAGCAGCTCGG-3′. The thermal cycling profile was: 35 cycles of 94°C for 1 min, 55°C for WAP, 53°C for CSN2, 62°C for RAMP3 and TBRG4 for 1 min and 72°C for 1 min, with a final extension of 72°C for 7 min.

DNA PROBES

The probes used for DNA FISH were: BAC RP23-225I14 (a kind gift from M. Rijnkels) containing a region of 204 kb including the *TBRG4*, *WAP*, and *RAMP3* genes, BAC RP23-457P20 (a kind gift from M. Rijnkels) carrying a 180 kb region including the casein genes *CSN1s1*, *CSN2* and *CSN1s2a*, two P1 vectors carrying the HS2 of the mouse globin LCR and the coding region of the embryonic Ey globin gene (a kind gift from M. Groudine, F. Hutchinson Cancer Res. Center, Seattle and C. Francastel, I. Cochin, Paris, France [Ragoczy et al., 2006]). Chromosome territories were labeled with biotinylated whole chromosome paints for MMU5, MMU7 and MMU11 (Cambio), and centromeres with mouse pan-centromeric probe-FITC (Cambio).

BAC probes were labeled with digoxigenin (DIG) using the DIG-High Prime system (Roche) or with biotin using the BioPrime® DNA labeling system (Invitrogen).

TWO-DIMENSIONAL DNA FISH

HC11 and 3T6 nuclei were isolated in 0.25% KCl/0.5% NaCitrate and fixed with 3:1 v/v methanol-acetic acid. They were spotted on superfrost slides and dried. The nuclei were then incubated with 200 μg/ml RNase in 2× SSC at 37°C for 30 min, washed in 2× SSC, and dehydrated through an ethanol series (70%, 90%, 100%). For denaturation, slides were incubated for 1 min 40 s in 70% formamide/2× SSC at 70°C, then cooled down in ice-cold 70% ethanol and dehydrated, as described above.

Fifty nanograms of BAC probes labeled with DIG were precipitated with 7 μg mouse Cot-1 DNA (Invitrogen) and 5 μg salmon sperm DNA. The probes were then resuspended in 13 μl of a

hybridization mix (Cambio), and 2 μl of biotinylated whole mouse chromosome paints (Cambio) were added. The probes were denatured at 95°C for 5 min, and then preannealed at 37°C for 20 min. Hybridization was performed overnight at 37°C in a humidified chamber. Following hybridization, the slides were washed three times in 2× SSC at 45°C for 4 min, three times in 0.1× SSC at 60°C for 4 min, and once in 4× SSC/0.1% Tween-20 at RT for 5 min. The slides were blocked in 4% BSA/4× SSC/0.1% Tween-20 at RT for 30 min.

DIG-labeled probes were detected with sheep anti-DIG TRITC (Roche) followed by donkey anti-sheep TRITC (FluoProbes®); biotinylated probes were detected with goat anti-biotine FITC (Vector Laboratories). Incubations with antibodies were performed in 4% BSA/4× SSC/0.1% Tween-20 at 37°C for 30 min. The slides were washed three times in 4× SSC/0.1% Tween-20 for 3 min at 37°C. The nuclei were then counterstained with DAPI (1 μg/ml) and mounted in Vectashield.

3D DNA FISH

After the induction of milk protein gene expression, the cells were trypsinized (230,000 cells/ml suspension) and cultured for 2 h or longer to allow for attachment on glass coverslips. In order to preserve their 3D structure, the following protocol was performed: cells were permeabilized for 5 min in ice-cold cytoskeletal buffer (0.5% Triton X-100, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 10 mM PIPES pH 6.8) prior to 4% PFA fixation for 10 min. The cells were then incubated in 0.1 N HCl for 5 min at RT and the RNA was removed with 200 μg/ml RNase in 2× SSC at 37°C for 30 min. The cells were kept in 50% formamide/2× SSC until hybridization. Probes including a 100-fold excess of sonicated mouse DNA and salmon sperm DNA were denatured at 75°C for 10 min and preannealed at 37°C for 90 min. The cells were then denatured, as described above, at 75°C for 3 min. Hybridization was performed at 37°C for 24 h. The cells were washed once in 50% formamide/2× SSC at 45°C for 3 min, four times in 2× SSC at 45°C for 3 min, four times in 0.1× SSC at 60°C for 3 min, and then blocked twice in 0.12% BSA/1× PBS/0.1% Tween-20 at RT for 10 min.

Detection was performed for 1 h at 37°C. DIG-labeled probes were detected with mouse anti-DIG Cy5 followed by sheep anti-mouse Cy5 (Jackson ImmunoResearch), biotin-labeled probes being detected as above. The nuclei were counterstained with propidium iodide (1 μg/ml) or DAPI (1 μg/ml) and mounted in Vectashield.

IMAGE ACQUISITION AND PROCESSING

CT images were taken on a Leica DMRB epifluorescence microscope (×100/NA 1.4 objective) equipped with a DP50 imaging camera (Olympus). These images were analyzed using ImageJ software [Abramoff et al., 2004]. For 3D analyses, a Zeiss LSM 510 confocal microscope (MIMA2 Platform, INRA) (×63/NA 1.4 objective) and an optical sectioning microscope attached to an AxioObserver imaging Apotome system (Zeiss) (×63/NA 1.4 objective) were used. Z stack images were acquired at intervals of 0.24 μm on three channels (nucleus, CSN spots and WAP spots) and saved as lsm files.

Automated 3D image processing and analysis was ensured using the ITK library (http://www.itk.org) interfaced with Python script language. The Python script implementing the whole procedure is available at the http://voxel.jouy.inra.fr/darcs/agrobi/. Nuclei were segmented using a threshold determined from the gradient of gray levels [Kittler et al., 1985]. A transformation to fill any dark holes [Soille, 2003] was performed before threshold determination in order to avoid threshold values that would be excessively high. Two different methods were used for spot detection: when images revealed a low level of noise, the top-hat filtering of sizes was ensured in order to homogenize the background prior to spot selection, applying a fixed threshold level. When antibody combinations yielded a higher level of noise, spot segmentation was performed by retaining the four largest and brightest spots in the nucleus [Vachier, 1997; Najman and Couprie, 2006]. All segmentations were checked and validated. Data files containing measurements such as centroid coordinates or eroded volume fractions (EVF) (see below) were corrected for nearby spots (which had not been separated), missing spots and false spots.

STATISTICAL SPATIAL ANALYSIS

Differences between localizations with respect to CTs were assessed using chi-square homogeneity tests.

The locations of CSN and WAP spot centroids with respect to the nuclear membrane were measured by their EVF. The EVF of a point within the nucleus is defined as the fraction of nuclear volume lying between a considered point and the nuclear membrane. The EVF rises from 0 at the nuclear periphery to 1 at the nuclear center. The EVF of points uniformly distributed within a nucleus is uniformly distributed between 0 and 1. This property holds for nuclei of any size and shape. It should be noted that the EVF changes more rapidly near the nuclear periphery than in the nuclear center. For instance, in a spherical nucleus with a radius of 5 μm, a point with an EVF equal to 0.5 lies only about 1 μm from the nuclear membrane. Standard erosion analyses [Parada et al., 2004a] were based on a discretized version of the EVF. EVFs were computed based on a Euclidean distance transform [Maurer et al., 2003].

An assessment of gene differences and stimulation effects on EVF was made using *F* tests. Other potential effects such as nucleus size (diameter) and flatness were also tested.

RESULTS

THE NUCLEAR LOCALIZATION OF MILK PROTEIN GENES IS NOT CELL TYPE-SPECIFIC BUT MODIFIED BY LACTOGENIC HORMONES

In order to clarify the mechanisms regulating milk protein gene expression, we investigated the nuclear positioning of *WAP* and *CSN* genes in HC11 cell nuclei. Dual color 3D FISH experiments were performed on nuclei from two independent HC11 cell preparations, using BAC probes containing the genes of interest. After FISH, individual nuclei were imaged by confocal microscopy, and the eroded volume fraction (EVF) was calculated for each signal. Because the HC11 cell line has a duplicated genome [Poussin et al., 2005] we analyzed four alleles per gene and per nucleus (i.e., eight signals per nucleus) (Fig. 1A).

We first of all analyzed HC11 cells cultured in the absence of lactogenic hormones and therefore not expressing milk protein genes (Fig. 1B). The EVF revealed a different positioning for *WAP* (mean EVF = 0.68) and *CSN* (mean EVF = 0.23) genes with respect to

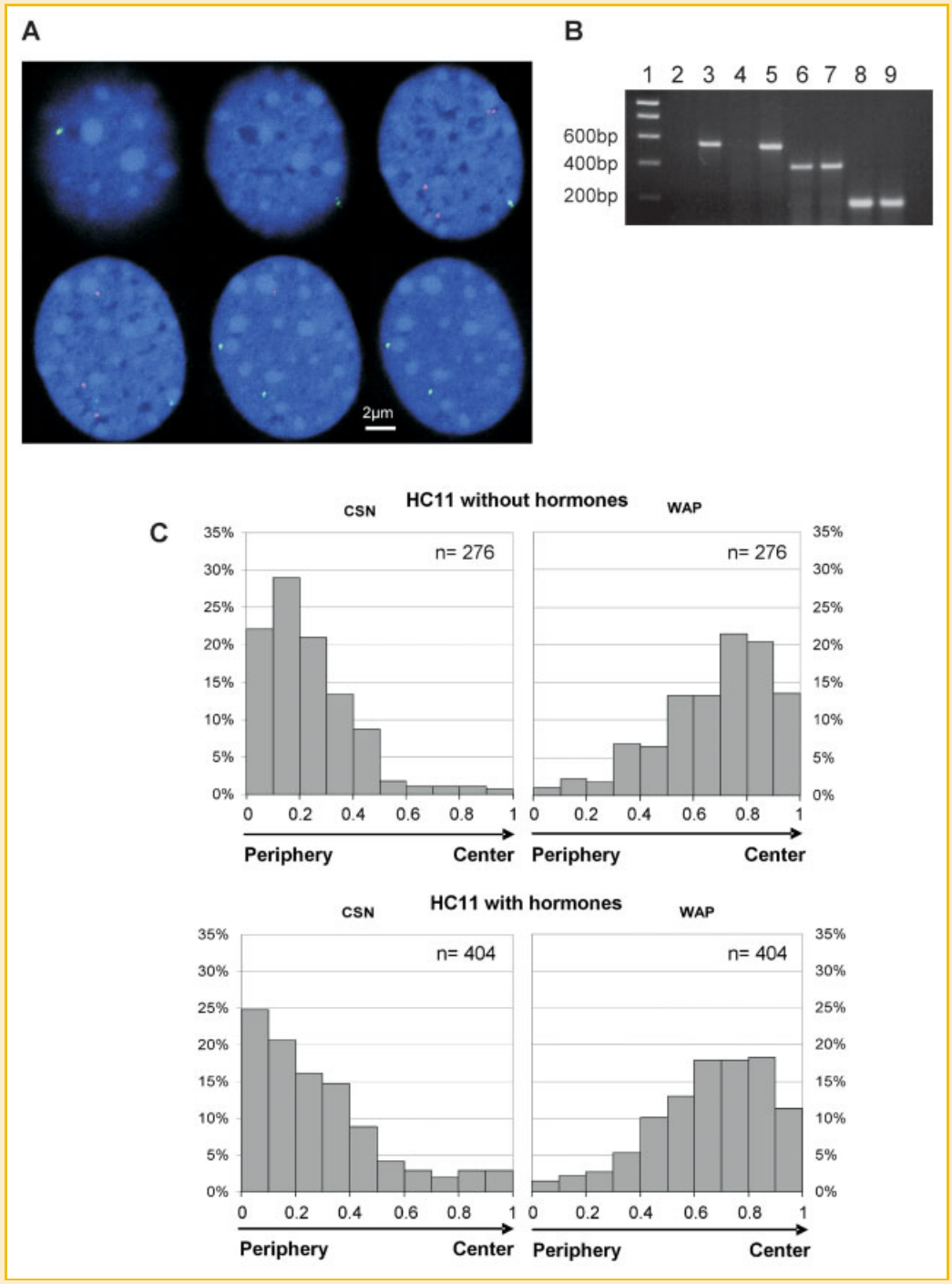

Fig. 1. A: Nuclear localization of *WAP* (red signals) and *CSN* (green signals) genes in the HC11 nucleus, indicating their differing distribution. Different slices of the same nucleus are shown. Nuclei were counterstained with IP (blue). B: Analysis of mRNA levels in HC11 cells cultured in the absence (lanes 2, 4, 6, 8) and presence (lanes 3, 5, 7, 9) of hormones: Lane 1, molecular size marker (SmartLadder, Eurogentec). Lanes 2 and 3, RT-PCR for *WAP* gene. Lanes 4 and 5, RT-PCR for gene *CSN2*. Lanes 6 and 7, RT-PCR for gene *TBRG4*. Lanes 8 and 9, RT-PCR for gene *RAMP3*. Negative controls of RT and PCR are not shown. C: EVFs for *CSN* and *WAP* genes in 3D nuclei from HC11 cells cultured with and without hormones. N = number of spots analyzed. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the nuclear periphery. Both genes were distributed non-uniformly within the nucleus: while *CSN* genes were located close to the nuclear periphery (94% of alleles with an EVF between 0 and 0.5), the *WAP* gene was detected in the center of the nucleus (55% of alleles between 0.7 and 1) (Fig. 1C). These results demonstrated a non-random distribution of *WAP* and *CSN* genes in HC11 cells that could be important to their functional regulation, and because they were differentially positioned, it could be expected that associations with different compartments of the nucleus might regulate their transcription.

WAP and *CSN* gene expression was then induced in HC11 cells by the addition of lactogenic hormones. Induction was checked by RT-PCR (Fig. 1B). Hormone stimulation affected mean EVF values for both genes (mean EVF values of 0.66 and 0.28 for *WAP* and *CSN* genes, respectively; *F*-test $P = 2\%$) and this effect was gene-specific (*F*-test $P = 0.4\%$). *CSN* and *WAP* genes moved slightly inwards (85% of alleles with an EVF of between 0 and 0.5) and outwards (47% of alleles between 0.7 and 1), respectively (Fig. 1C). Thus the induction of milk protein gene transcription by lactogenic hormones was correlated with a nuclear repositioning of genes that could be important to optimizing their transcriptional activity. These movements, which are of an inverse nature, are probably not due to a global reorganization of the nucleus.

After hormonal stimulation we also found significant differences in the nuclear shape of HC11 cells. Nuclei from HC11 cells cultured in the presence of lactogenic hormones had a smaller diameter (*F*-test $P = 0.14\%$) and were flatter (*F*-test $P = 0.082\%$) than those from HC11 cells cultured without lactogenic hormones. However, we did not find any relationship between mean EVF values of *CSN* and *WAP* genes and nuclear diameter and flatness (*F*-test $P = 41\%$) that could account for the different positioning of genes.

Next, in order to determine whether *WAP* and *CSN* gene distribution was specific to HC11 cells, similar experiments were performed in 3T6 cells which do not express milk protein genes. We found the same gene distribution as in HC11 cells (data not shown), the *CSN* gene being located close to the nuclear periphery and the *WAP* gene being more central; we therefore reached the conclusion that milk protein genes are non-randomly positioned, although their distribution is not specific to mammary epithelial cells.

WAP AND CSN GENES DO NOT FREQUENTLY ASSOCIATE WITH EACH OTHER IN HORMONE INDUCED CELLS

Interchromosomal associations between genes that are regulated in a coordinated fashion have been described in a variety of cell types [Spilianakis et al., 2005]. Because the expression of milk protein genes is induced by several common transcription factors, we were interested in determining whether *WAP* and *CSN* genes are associated in specific nuclear regions which could facilitate their co-expression.

We measured the distances between each WAP and CSN allele and its closest homologous and heterologous genes in HC11 cells cultured in the absence or presence of lactogenic hormones. Signals were scored as being juxtaposed or colocalized when the distance between them was less than 1 μm [Osborne et al., 2004; Brown et al., 2006]. The percentage of signals in this case was lower than 2.3% for both homologous and heterologous pairs, there being no significant difference after hormonal induction and activation of the genes. Thus the different alleles of *WAP* and *CSN* genes were not frequently found in close vicinity, even though they share the same regulatory mechanism.

POSITIONING OF *WAP* AND *CSN* GENES WITH RESPECT TO THEIR CHROMOSOME TERRITORIES

The relocation of genes outside CTs has been described when their expression is induced [Volpi et al., 2000; Williams et al., 2002; Chambeyron et al., 2005].

To address the possibility that the transcriptional activation of *WAP* and *CSN* genes correlates with their position in their CT, we examined the location of these genes with respect to their CT (11 and 5, respectively) in HC11 cells with or without hormone treatment. 2D FISH experiments for each gene and CT were performed on methanol-acetic acid fixed nuclei from two different HC11 cell preparations. The positioning of genes with respect to their CT was scored in four categories (Fig. 2A).

Different results were found for *CSN* and *WAP* genes. In cells not stimulated by hormones and thus not expressing milk protein genes, we found a higher frequency of WAP signals localized outside the CT (29%) when compared with the frequency of external CSN signals (13%). Furthermore, although a significantly increased proportion of external signals (24%) was found for *CSN* genes relative to CT5 in HC11 cells cultured in the presence of lactogenic hormones (Pearson's Chi-squared test; $P = 0.07\%$), no differences were found in the percentages of external WAP signals with respect to their CT11 in HC11 cells with or without hormones (Table I). As a control, we also studied a non-milk protein gene, the β-*globin* gene, the regulation of which is not sensitive to lactogenic hormones. A probe located within the β-*globin* locus generated signals that were mainly located within the expected CT (MMU7) in cells cultured without hormones (72% of signals). Signal positions relative to the CT were not significantly different in induced cells (74% of signals inside the CT).

To further our studies, we performed the same experiments in 3T6 cells that did not express milk protein genes. The distribution of *WAP* and *CSN* gene signals relative to their CT in 3T6 cells did not differ from that found in non-induced HC11 cells (Table I).

Taken together, these results did reveal that the position of *CSN* genes relative to their CT was correlated with lactogenic hormone induction and thus with their transcriptional activation. However, the results differed for the *WAP* gene. The *WAP* gene loops out from its CT, whether it is transcribed or not. The mouse WAP gene is located between two genes (*TBRG4* and *RAMP3*) (Fig. 2A) which are expressed in different tissues, including the mammary gland. RT-PCR analysis was performed to study the expression of these genes in HC11 cells cultured in the presence and absence of hormones. Our findings confirmed the expression of *TBRG4* and *RAMP3* genes in HC11 cells (Fig. 1B) and suggested that the chromatin loop containing these transcribed flanking genes allowed the positioning of the *WAP* gene outside its CT even when the *WAP* gene was not transcribed. Another mechanism controlling *WAP* transcription regulation may be involved.

POSITIONING OF *WAP* AND *CSN* GENES WITH RESPECT TO CENTROMERIC HETEROCHROMATIN

The transcriptional regulation of several genes has been related to their association with other nuclear compartments rather than their positioning relative to the CT [Zink et al., 2004]. Because the physical localization of loci near the centromeric heterochromatin contributes to their silencing, we next studied the positioning of the *WAP* gene (which is located near the centromere (0.5 cM) in chromosome MMU11 (Fig. 2C)), and the *CSN* gene with respect to the centromeric heterochromatin. In mouse interphase nuclei, centromeric and pericentromeric regions cluster to generate

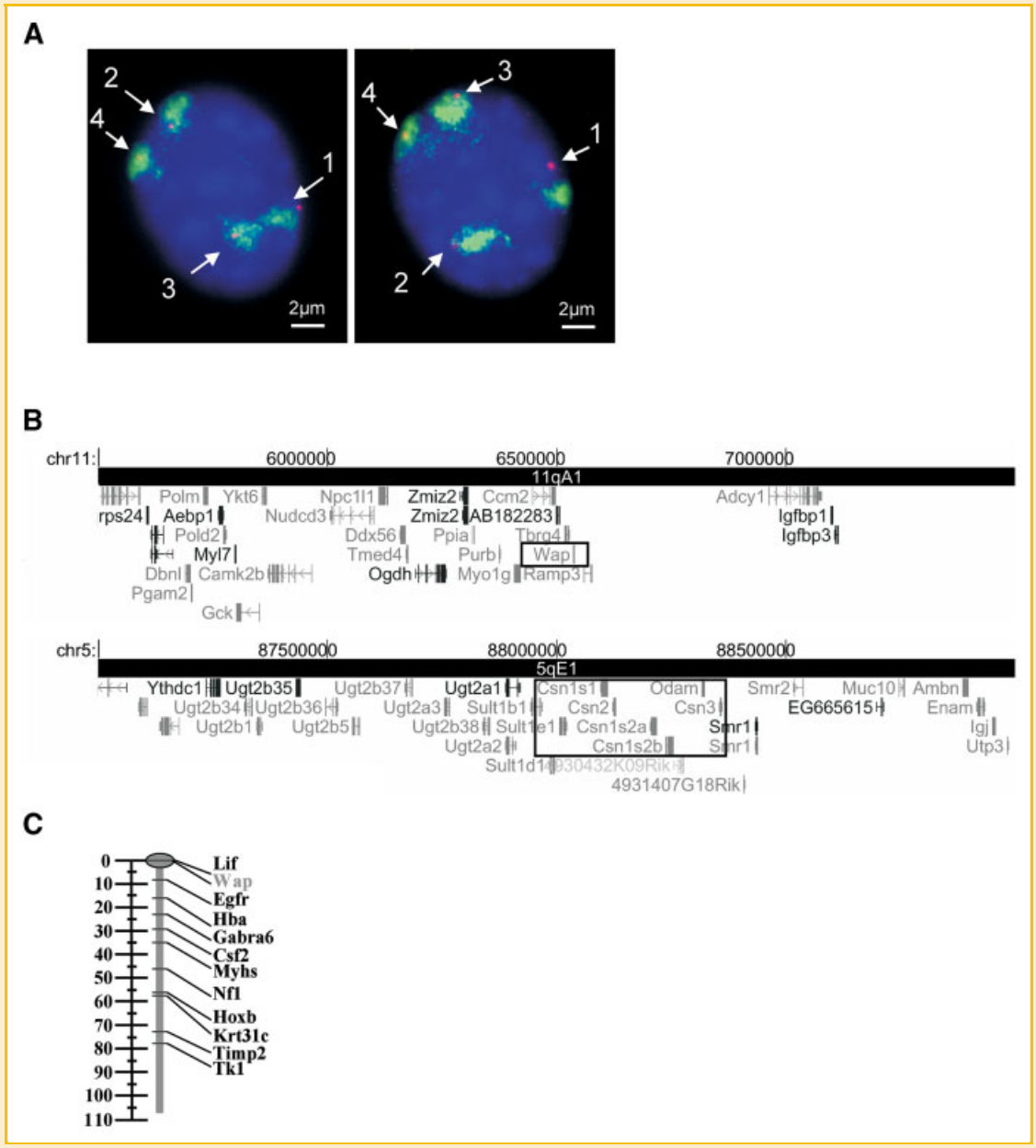

Fig. 2. A: Positioning of *CSN* and *WAP* genes (red) with respect to their chromosome 5 and 11 territories, respectively (green). Signals were scored in four different categories: (1) localization outside, (2) at the edge-outside, (3) at the edge-inside, or (4) inside the chromosome territory. B: Genomic maps showing 2 Mb surrounding regions of *CSN* and *WAP* genes on MMU5 and 11, respectively (UCSC Genome Browser on Mouse July 2007 Assembly, http://genome.ucsc.edu/cgi-bin/hggateway). C: *WAP* gene positioning on the Mouse Chromosome 11 Linkage Map (http://www.informatics.jax.org/). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I. Percentages of *WAP* and *CSN* Genes Scored Outside (O), at the Edge-Outside (EO), at the Edge-Inside (EI) or Inside (I) Their Chromosome Territories (CT) in HC11 and 3T6 Cells

Genes	Chromosome MMU	Cell type	No. of CT observed	O (%)	EO (%)	EI (%)	I (%)
CSN	5	HC11 non-induced	339	13	34	44	9
CSN	5	HC11 induced	249	24	36	31	9
CSN	5	3T6	131	11	31	40	19
WAP	11	HC11 non-induced	189	29	40	28	4
WAP	11	HC11 induced	284	25	37	33	6
WAP	11	3T6	150	22	45	29	3

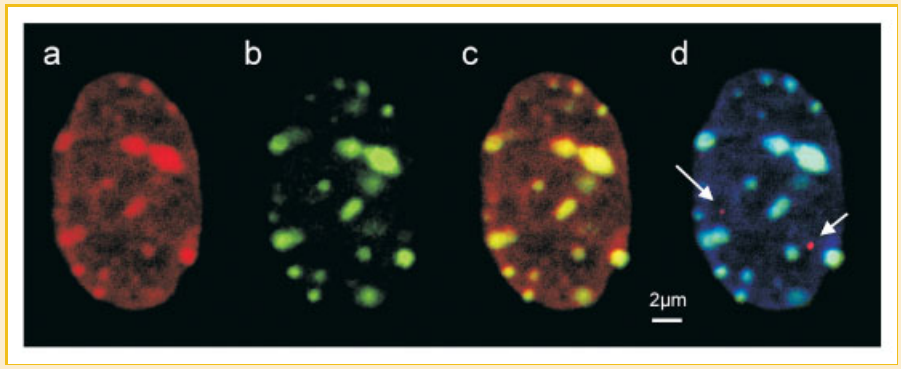

Fig. 3. Positioning of milk protein genes relative to centromeric DNA clusters in the nucleus of HC11 cells. Images of a representative HC11 nucleus (a–d): DNA counterstaining (DAPI) is shown in red (a), mouse chromocenters are shown in green (b), the overlap of both signals in chromocenters is shown in yellow (c), the two *CSN* gene signals not-associated with chromocenters are indicated by arrows (d). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

chromocenters that can be easily identified by counterstaining the nuclei of cells with DAPI [Mayer et al., 2005]. We first performed 3D FISH experiments using a commercial mouse pan-centromeric probe (Cambio) to verify that the FISH signals colocalized with dense foci of DAPI (Fig. 3). After 3D FISH experiments with specific probes for each gene, the loci were scored as being associated (when they were in contact or colocalized with DAPI foci) or dissociated (in the contrary situation) (Fig. 3). No significant differences were found between CSN or WAP signals relative to chromocenters under both HC11 conditions. Moreover, a high percentage of signals (70%) was found not to be associated with chromocenters, suggesting that the centromeric heterochromatin does not play a critical role in the regulation of *WAP* and *CSN* genes (Table II).

DISCUSSION

Lactogenic hormones play an important role in the transcriptional activation of milk protein genes, inducing chromatin modifications [Millot et al., 2003] and the association of transcription factors with regulatory regions of milk protein genes [Kabotyanski et al., 2006; Xu et al., 2007]. Because the positioning of mammalian gene loci within cell nuclei is not random and has been linked to their activity, our aim was to determine whether the localization of milk protein genes within the mammary epithelial cell nucleus was also related to their transcriptional regulation.

TABLE II. Association of *CSN* and *WAP* Genes With Chromocenters in HC11 Cells Cultured in the Absence or Presence of Lactogenic Hormones

	Gene or locus[a]	
HC11 cells	*CSN*	*WAP*
Non-induced (number of nuclei observed)	49	57
Associated (%)	25	29
Not associated (%)	75	71
Induced (number of nuclei observed)	57	28
Associated (%)	28	33
Not associated (%)	72	67

[a]Four signals per nucleus were found because HC11 cells have a duplicated genome.

Our results demonstrate that the transcriptional activation of *WAP* and *CSN* genes by lactogenic hormones is coupled to changes in their nuclear organization. In non-induced cells, non-random milk protein gene locations (*CSN* gene on the outside and *WAP* gene more centrally) seemed to be in accordance with the position of their relative CTs. The arrangement of chromosomes in the interphase cell nucleus is not random and has been linked to chromosome size and gene density [Parada et al., 2004b; Mayer et al., 2005]. Previous studies in six mouse cell types determined the cell type-specific radial distribution of CTs. In these cells, the distribution of the most gene-rich chromosome MMU11 was found to be more internal than all the other chromosomes studied [Mayer et al., 2005]. In HC11 cells, the *WAP* gene mapped on chromosome MMU11 is also located in a more internal position than the *CSN* gene mapped on chromosome MMU5. Parada et al. [2004a] described a variable distribution of chromosome MMU5 in several mouse tissues, suggesting that the overall transcriptional activity of its genes could also determine its nuclear arrangement. In HC11 3D nuclei, we found that CT5 was mostly situated at the nuclear periphery (data not shown), a localization consistent with the peripheral position of *CSN*.

In 2004, Zink et al. described the association of *GASZ*, *CFTR*, and *CORTBP2* genes with the nuclear periphery and pericentric heterochromatin in their inactive states. They suggested that as the layer of perinuclear heterochromatin is relatively thin, distances between inactive genes and the nuclear envelope were <1 μm. Based on our EVF analysis, we are able to evaluate gene distribution relative to the nuclear periphery in 3D nuclei and also detect any small differences in gene position. A high percentage of inactive CSN alleles was found near the nuclear periphery (<1 μm), suggesting a possible role for this nuclear compartment in *CSN* gene repression. When milk protein gene transcription was induced, the *CSN* gene moved towards the interior of the nucleus, a re-localization that could facilitate its transcription. However, the inactive *WAP* loci that were located in the interior of the nucleus moved towards the periphery, suggesting that another mechanism regulated their transcription. These movements, which are of an

inverse nature, are probably not due to a global reorganization of the nucleus.

When we next studied the position of *WAP* and *CSN* genes with respect to their CTs in order to determine whether chromatin loops unfolded when the genes were transcribed, different results were also found. The position of the *CSN* gene with respect to CT5 correlated with its transcriptional status. Similar results have been described elsewhere for different gene clusters containing structurally and functionally related genes. When their gene expression is induced, MHC and EDC form loops extending from CT 6 and 1, respectively [Volpi et al., 2000; Williams et al., 2002; Chambeyron et al., 2005]. Our results suggest that the chromatin loop containing the *CSN* locus—which corresponds to a family of five, coordinately regulated genes (Fig. 2B)—relocates outside its CT when transcription is activated by lactogenic hormones. In contrast to *CSN* genes, *WAP* gene localization with respect to its CT11 does not change in line with its transcriptional status. Moreover, we found a high frequency of *WAP* genes sited at distance from their CTs in HC11 cells in the absence of hormones or in fibroblasts where the *WAP* gene is not expressed. Previous studies had shown that the positioning of certain genes relative to their CTs is more closely determined by their chromosomal context that their transcriptional status. While human *α-globin* genes (which are located in a gene-dense region near the telomere) frequently localize away from their territories irrespective of their transcriptional status, mouse *α-globin* genes are most frequently positioned inside their territories [Brown et al., 2006]. It seems that the location of mouse *α-globin* genes close to the chromosome MMU11 centromere in a region of lower gene density is critical to determining the positioning of these genes inside their CT [Brown et al., 2006]. However, the *WAP* gene which maps nearer to the centromere (band 11qA1, 0.5 cM), than *α-globin* genes (11qA4, 16 cM), and is close to a gene-poor region, localizes outside its CT11 independently of its transcriptional status. The *WAP* gene is located between two genes that are expressed in several tissues, including the mammary gland. Our results suggest that neighboring transcriptional activity rather than chromosome localization is important to determining the position of the *WAP* gene outside its territory. These findings are in line with the high frequency of WAP alleles that did not associate with chromocenters in HC11 cells, suggesting that the *WAP* gene is located in an active chromatin region that moves away from the neighboring centromeric heterochromatin.

Previous studies by our group had described variations in the chromatin structure surrounding the mouse and rabbit *WAP* gene in mammary gland tissue [Millot et al., 2003; Montazer-Torbati et al., 2008]. During gestation, the upstream chromatin structure is only partially opened, limiting the interaction of transcription factors with their regulatory sequences. At the end of pregnancy and during lactation, when lactogenic hormones induce the full activation of transcription factors, the chromatin structure adopts an open conformation allowing access for transcription factors to their target sequences. Because the position of the *WAP* gene in HC11 cells is related to its chromosome context, local mechanisms intervening in the chromatin structure, rather than association with nuclear compartments, seem to be more critical for WAP gene regulation.

When transcription is induced, active genes on decondensed chromatin loops unfold from their CTs to colocalize both *in cis* and *in trans* at transcription factories [Osborne et al., 2004, 2007]. Further studies are necessary to determine the association between *CSN* and *WAP* genes and transcription factories. However, insofar as these genes were not frequently found near to each other when their transcription was induced, associations with different transcription factories can be expected.

In summary, the nuclear positioning of *WAP* and *CSN* genes in HC11 cells is related to their transcriptional status. Moreover, the different chromosome contexts of these genes play an important role in their regulatory mechanisms and also in their association with nuclear compartments. Since HC11 cells are derived from mid-pregnant mice, these cells are not fully representative of terminally differentiated mammary epithelial cells. However, as they have retained their capacity to respond to lactogenic hormones, they can be used as a model that reflects the activation and re-positioning of milk protein genes in the nucleus after hormonal treatments. Previous studies in human mammary epithelial cells revealed the importance of the extracellular matrix to nuclear architecture [Lelievre et al., 1998; Le Beyec et al., 2007].

Even though the 3D culture of immortalized mammary cells on extracellular matrix induces a response to lactogenic hormones which is more similar to physiological conditions, such cells are very different from the normal mammary epithelial cells observed during correct differentiation in vivo. We have therefore decided that our next studies will focus on what happens in the mammary gland in vivo. Preliminary experiments are tending to show that the localization of WAP and CSN genes in the HC11 model is in line with that observed in vivo.

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