Journal of Cellular Biochemistry 96:611–621 (2005)

Interactions Between the Rabbit *CSN1* Gene and the Nuclear Matrix of Stably Transfected HC11 Mammary Epithelial Cells Vary With Its Level of Expression

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Abstract The expression of casein genes is specific to the mammary gland and maximal during lactation. However, among the numerous mammary cell lines described so far, only a few express some casein genes. The regulatory regions of casein genes have been largely described but the mechanisms explaining the mammary specific expression of these genes, and their silencing in most mammary cell lines, have not yet been fully elucidated. To test the hypothesis that the nuclear location of the casein genes may affect their expression, we transfected HC11 mouse mammary cell line with a 100 kb DNA fragment surrounding the rabbit alpha S1 casein gene. We derived stable clones which express or not the transfected rabbit casein gene, in the same cellular context, independently of the number of transgene copies. Metaphase spreads were prepared from the different clones and the transfected genes were localized. Unexpectedly, we observed that in the original HC11 cell line the number of chromosomes per metaphase spread is close to 80, suggesting that HC11 cells have undergone a duplication event, since the mouse karyotype is $2n = 40$. In alpha S1 casein expressing cells, the expression level does not clearly correlate with a localization of the transfected DNA proximal to the centromeres or the telomeres. Analysis of the localization of the transfected DNA in nuclear halos allows us to conclude that when expressed, transfected DNA is more closely linked to the nuclear matrix. The next step will be to study the attachment of the endogenous casein gene in mammary nuclei during lactation. J. Cell. Biochem. 96: 611–621, 2005.

Key words: HC11 cells; nuclear halos; transcription; bacterial artificial chromosome; milk

Many recent studies have focused on the impact of the subnuclear location of a gene on its expression [Pederson, 2004]: transcriptionally active genes are early replicated and located more in the central part of the nucleus, whereas non active sequences are replicated later and located essentially in the periphery of the nucleus and/or the nucleolus [Isser et al., 1998; Sadoni et al., 1999; Zink et al., 1999]. In addition, active genes seem to be preferentially associated with the nuclear matrix, whereas inactive genes would be essentially located in chromatin loops which are freely extruded out of the nucleus after nuclear extraction of chromatin bound protein, as shown in "nuclear halos" preparations [Gerdes et al., 1994; Iarovaia et al., 2004].

The mammary gland is a complex tissue in which the mammary epithelial cells (MEC) can synthesize and secrete huge amounts of milk proteins, mainly composed of the caseins (CSN) (alpha S1 casein: CSNS1, alphaS2 casein, beta casein: CSNS2, and kappa casein: CSN3). This production is regulated to restrict it to the lactation period and is specific to the MEC. *CSN* genes are thus highly regulated, being expressed at the highest levels in the mammary gland during lactation, and not being expressed at significant levels at other times [Puissant et al., 1994]. Several mammary cell lines have

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Received 11 March 2005; Accepted 17 May 2005
DOI 10.1002/jcb.20560

been isolated. However, none of the tumor cell lines described so far can express milk protein genes in the presence of lactogenic hormones, whereas a few immortalized cell lines have been reported to express the *CSN2* gene [Ball et al., 1988]. In the latter, *CSN1* and *CSN3* genes are not expressed to significant levels [Kolb, 2002]. The molecular mechanisms of this tight regulation of milk protein genes have not been fully elucidated, and nuclear repositioning of these genes with respect to the overall nuclear topology and nuclear matrix may be involved in both mammary gland differentiation and casein gene expression in immortalized mammary cells.

In an attempt to clarify these mechanisms, we have produced cellular clones derived from an immortalized mouse mammary cell line (HC11) [Ball et al., 1988] transfected with the rabbit *CSN1* gene [Pauloin et al., 2002]. Different clones, expressing the rabbit *CSN1* gene to different levels, have been isolated. The rationale of the present study was to determine whether a correlation exists between the expression level of the *CSN1* gene and its nuclear matrix attachment. This will constitute the first step on the road to study a more physiological model, i.e., the mammary gland during different physiological states.

MATERIALS AND METHODS

HC11 Cell Culture and Transfection

HC11 cells were grown in RPMI 1640 medium containing 5% fetal calf serum (HyClone, Perbio) and 4 μg/ml insulin. They were transfected with two BAC clones, isolated from a rabbit genomic DNA library [Rogel-Gaillard et al., 2001] harboring CSN1 and part or all of *CSN2* genes (243C12 and 746F5, respectively) [Pauloin et al., 2002] and pSV2 neo [Gorman et al., 1983] DNA (10 and 2 μg per 10 cm plate, respectively), using lipofectAMINE (20 μl) as recommended by the manufacturer (Life Technologies, Cergy-Pontoise, France). Transfected cells were selected in the presence of geniticin (300 μg G418/ml) as previously described [Doppler et al., 1989]. Isolated clones of resistant cells were harvested and grown independently. When necessary, they were kept frozen until use.

To promote differentiation, confluent population of cells were kept for 2 days in RPMI 1640 medium containing 10% horse serum, 4 μg/ml insulin, and 1 μM cortisol, and then milk protein gene expression was induced for 4 days in the same medium supplemented with 5 μg/ml prolactin [Pauloin et al., 2002].

Northern and Southern Analyses, Real Time PCR, and RT-PCR

DNA was extracted from HC11 cells and analyzed on Southern blots, as previously described [Hiripi et al., 1998]. RNA was extracted, analyzed on Northern blots and reverse transcribed into cDNA, as previously described [Hiripi et al., 1998]. Hybridization signals were quantified using a STORM™ and the ImageQuant™ software (Molecular Dynamics Inc., Sunnyvale, CA).

Real time PCR performed in 25 μl assays, using the qPCR™ Core kit, Sybr Green I solution and Uracil DNA Glycosylase from Eurogentec, France, in accordance with the manufacturer's recommendations. For mouse CSN2 cDNA or gene amplifications, the primers were 5′-AGCCCCAGGCCTTTCCATA-3′ and 5′-TCAGGAGAAATGACAGGCCC-3′, for rabbit CSN1 cDNA or gene amplifications, the primers were 5′-TTCAGCCTTTCGAACAACCCT-3′ and 5′-ACGTCAGTTTTTTCAGCACTCTC-3′ and for mouse cyclophilin cDNA amplifications the primers were 5′-TCCATGGCTTCCACAATGTT-3′ and 5′-CATCCTAAAGCATACAGGTCCTG-3′. In parallel to the samples, serial dilutions of mouse CSN2 and rabbit CSN1 cDNA plasmids (from 1.6×10^{-4} to 1.6×10^{-10} pmoles) were run to obtain calibration curves. The PCR were run on a Perkin Elmer Applied Biosystems 7700 apparatus and data was analyzed using the Sequence detection systems 1.6.3 software.

Chromosome Preparation for In Situ Hybridization

Metaphase spreads were obtained from transfected HC11 cells as already described [Hayes et al., 1991]. Briefly, cells were incubated with 3.3 mM thymidine overnight at 37°C, then washed three times with fresh medium devoid of thymidine and cultured for 4 h at 37°C in the presence of serum. Cells were then incubated with colchicine (0.04 μg/ml RPMI media) for 20 min at 37°C. After several washes in a medium containing serum, cells in metaphase were collected by a mild treatment with diluted trypsin (50 ng/ml), pelleted and suspended gently in an hypotonic solution containing 18%

v/v fætal calf serum and 0.18 mM EDTA. They were then maintained for 13 min at 37°C. A cold solution of methanol/acetic acid (3/1) was added drop by drop (total of 1 ml for 9 ml of cell suspension), and the resulting mixture was incubated for 5 min at room temperature. Nuclei were pelleted (650*g* for 10 min), washed once with the same cold solution, and resuspended in a small volume of methanol/acetic acid (3/1) adjusted after testing the metaphase density on a slide. To maximize the spreading of chromosomes, the suspension was dropped from a height of about 10 cm onto clean glass slides laid on wet tissue paper, and then air-dried before storage. The slides were stored at −20°C until use. Prior to in situ hybridization, the slides were treated with RNase A (100 μg/ml) at 37°C, and then sequentially washed at room temperature for 2 min with ethanol 70%, 80%, and 90%.

Nuclear Halo Preparation

The procedure was adapted from that described by Gerdes et al. [1994]: 1×10^6 cells were pelleted at 650*g* for 5 min and washed twice in PBS. The cells were then resuspended at a concentration of 1×10^6 cells/ml in an isotonic CSK1 buffer at pH 6.8 (10 mM pipes pH 6.8, 100 mM KCl, 0.3M sucrose, 3 mM $MgCl_2$, 1 mM EGTA) [de Belle et al., 1998] in the presence of 0.5% Triton X-100 and 0.5 mM phenyl methyl sulfonyl fluoride (PMSF) and incubated on ice for 15 min. 1×10^5 nuclei were then centrifuged onto coverslips for 5 min using a cytospin 2 cytological centrifuge (Shandon, Inc., Pittsburgh, PA). The coverslips were then rinsed twice in 1× PBS for 2 min followed by extraction of the soluble proteins in T2 buffer (10 mM pipes pH 6.8, 2M NaCl, 10 mM EDTA) [de Belle et al., 1998] for 12 min at room temperature. After several rinses in PBS and a brief dehydration in 10%, 30%, 70%, and 95% ethanol (2 min each, except for the last wash which was prolonged for 5 min), the coverslips were air-dried and nuclear halos were fixed by baking at 70°C for 2 h [Lawrence et al., 1988]. They were then stored overnight at 4°C prior to in situ hybridization.

Immunofluorescence Labeling of Nuclear Halos for Lamin A/C

In order to visualize the nuclear periphery, immunolabeling for lamin A/C was performed on nuclear halos, before in situ hybridization. All steps were performed at room temperature. Briefly, coverslips were saturated with PBS containing 3% BSA for 30 min and the immunofluorescence reaction was performed with a monoclonal mouse anti-lamin A/C antibody (monoclonal antibody R27, generous gift of G. Krohne, Wurzburg, Germany) diluted 50-fold in PBS containing 2% BSA for 1 h. After several washes in PBS, a donkey Texas Red coupled anti-mouse IgM antibody (Jackson Immuno Research, Baltimore) diluted 200-fold in PBS containing 2% BSA, was added for 1 h. The DNA was then stained with Hoechst 33342 (4 μg/ml in PBS for 20 min).

In Situ Hybridization

Preparation of probes. Approximately 2 μg of total DNA from BAC clone 243C12 were labeled with 11-dUTP digoxigenin (DIG), using a nick translation kit (Roche Diagnostics, Meylan, France). Labeled DNA was purified using a MicroSpin™ G-50 Column (Amersham Bioscience, Les Ulis, France). The purified DNA probe was mixed with a 100-fold excess of an equimolar mixture of unlabeled, sonicated, total rabbit DNA and herring sperm DNA. The DNA mixture was resuspended in a hybridization buffer (50% formamide, 2× SSC, 1× Denhardt, 10% of dextran sulphate, 40 mM NaH_2PO_4, pH 7) and kept at −20°C until hybridization.

Hybridization procedures. All slides (halos or metaphase spreads) were denatured in 70% formamide, 2× SSC pH 5.6 for 5 min at 72°C. The slides were sequentially rinsed at room temperature for 2 min with ethanol 70%, 80%, and 90%, and then briefly air-dried prior to hybridization.

The BAC DNA probe was denatured at 72°C for 5 min and then kept on ice for 10 min. For each slide, a quantity of DNA mixture containing approximately 300 ng of the specific probe was used for hybridization at 37°C for 24 h.

After hybridization, the slides were rinsed in a solution of 0.4× SSC pH 5.3 containing 0.3% Nonidet NP-40 for 2 min at 75°C and then for a few min in a 2× SSC pH 6.7, 0.1% NP-40 solution at room temperature. Several washes in PBS were then performed before immunodetection. The labeled probe was revealed with a sheep polyclonal anti-DIG antibody (Roche Diagnostics) diluted 200-fold in PBS containing 2% BSA, for 1 h at 37°C. Slides were then washed in PBS before a second reaction with a

donkey anti sheep IgG antibody coupled to Texas Red (Jackson Immuno Research) diluted 200-fold in PBS containing 2% BSA, for 1 h at 37°C. After several washes with PBS, the DNA was counterstained with Hoechst 33342 (4 μg/ml in PBS for 20 min at room temperature).

Fluorescence microscopy. Slides (nuclear halos and metaphase spreads), were observed on an inverted ZEISS AXIOVERT 35 microscope equipped for epifluorescence. Images were captured on a CCD camera (Photometrics KAF 1400) using a 100× objective, and then analyzed with the IPLab Spectrum software.

RESULTS

Different Populations of HC11 Transfected Cells Express the Rabbit *CSN1* Gene to Different Degrees

Two independent series of transfections made it possible to obtain geneticin-resistant HC11 cells. From these resistant cells, 49 clearly isolated cells were selected, grown independently and resulted in 27 clones, 18 in the first series and 9 in the second series of clones.

During the first series of transfections with BAC 243C12, in the presence of lactogenic hormones, only two of the 18 selected clones expressed the rabbit *CSN1* gene at levels which could easily be detected by Northern blot analyses. The result obtained with one of these clones (D8) is shown in Figure 1, right-hand upper panel: the mRNA migrating at the same position as that observed for rabbit mammary gland RNA and thus being of the size expected for the rabbit CSN1. The signal observed for 10 μg of total RNA, extracted from transfected HC11 cells, was similar to that observed for 0.1 μg RNA from lactating rabbit mammary gland tissue. From all the other cell populations, which either did not express the rabbit *CSN1* gene or only expressed it at low levels, cells from clone D2 were selected as being representative. These cells expressed the rabbit *CSN1* gene at very low levels. A signal corresponding to the expected size for rabbit CSN1 mRNA was barely detected on an overexposed Northern blot (Fig. 1, left-hand upper panel). Expression levels were evaluated precisely using real time RT-PCR (Table I) relative to mouse cyclophilin mRNA levels [Hasel and Sutcliffe, 1990]. Evaluation of CSN1 expression level in clone D2 and D8 showed that it was ninefold lower in D2 than in D8. These two clones, D2 and D8, are therefore referred to as expressing rabbit CSN1 to low and high levels, respectively.

During another series of experiments, nine populations of cells transfected with BAC 746F5 were selected, of which two expressed the rabbit CSN1 at levels detectable by Northern analyses. Two populations of cells: C5 (one of the two expressing cell populations) and C9 (one of the seven non-expressing cell populations) were further studied, as described above for D2 and D8 clones. Cells from clone C5 expressed the rabbit *CSN1* gene at a level between that seen for clones D2 and D8, and C9 expressed it at a lower level comparable to that seen for clone D2 (Table I).

Interestingly, in clones expressing higher levels of the rabbit *CSN1* gene, expression of the endogenous mouse *CSN2* gene was dramatically diminished and almost undetectable (Table I, Fig. 1, right panel), whereas it was clearly detected in other clones (Fig. 1, left-hand panel) as well as in non-transfected cells (data not shown), and reached intermediate levels in populations expressing intermediate levels of the rabbit CSN1 (data not shown). As previously described [Pauloin et al., 2002], even though the coding region of the rabbit *CSN2* gene was present in the BAC 243C12 transfected into the cells, it was not expressed, most likely because of the short length (around 1 kb) of the CSN2 5′ flanking region present in this BAC (Fig. 2A).

Level of Expression Is Not Related to the Rabbit *CSN1* Gene Copy Number

Genomic DNA was extracted from the different clones or from rabbit liver and analyzed on Southern blots after digestion with *Eco RI*. The blots were then hybridized with an almost full length rabbit CSN1 cDNA probe [Devinoy et al., 1988]. A similar pattern of three bands (8.5, 5.3, and 4 kb) was observed for both the transfected cells and the rabbit genomic DNA, showing that the large BAC DNA fragment had been integrated without any major rearrangement around the rabbit *CSN1* gene (Fig. 2B). For equivalent amounts of DNA, as shown by ethidium bromide staining, a very strong signal was detected for the DNA of D2 cells, whereas a weaker signal was observed with the other three cell populations. The transfected copy number was then evaluated using real time PCR. D2 cells were estimated to harbor 104 copies per diploid genome, whereas D8, C5, and C9 cells were estimated to harbor 46, 69 and 60

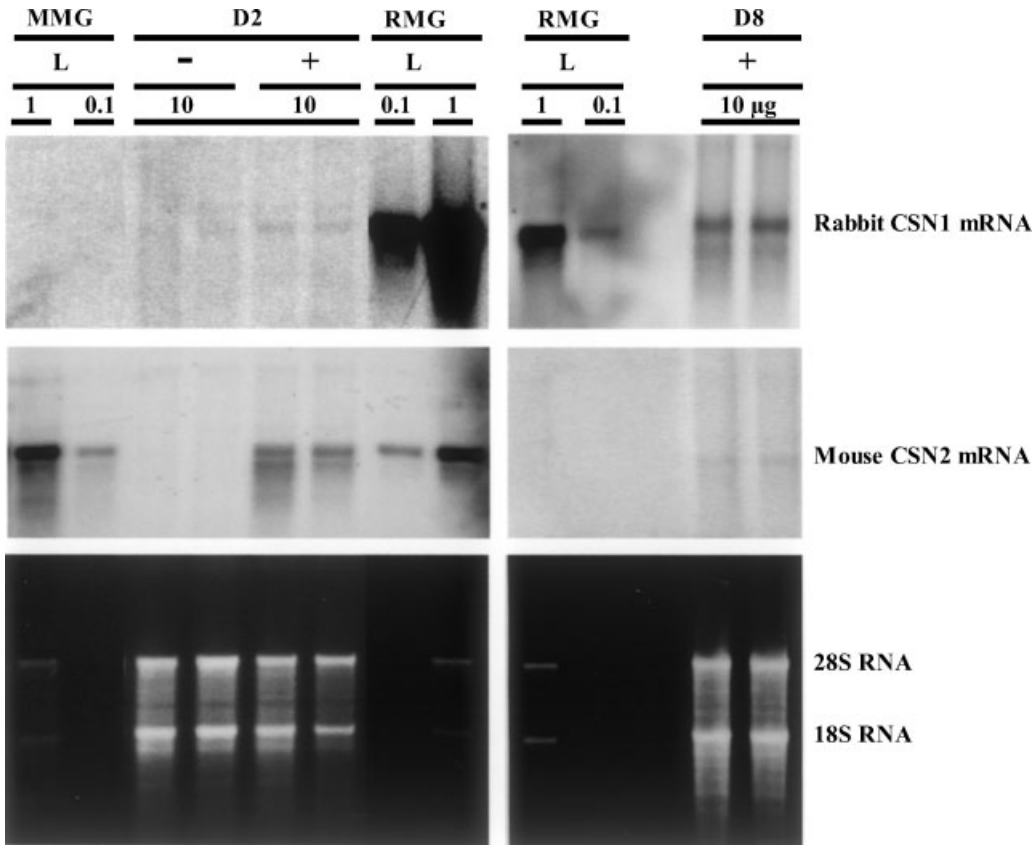

Fig. 1. Expression of rabbit *CSN1* gene in modified HC11 cells arising from different D2 or D8 clones which had been stably transfected with BAC 243C12 DNA containing the rabbit *CSN1* gene. Total RNA (10 µg), from transfected HC11 cells grown in the absence (−) or presence (+) of lactogenic hormones, were analyzed on Northern blots. Lactating (L) rabbit (RMG) or mouse (MMG) mammary gland total RNA (0.1 and 1 µg) were also loaded on each gel, as controls. For the D8 cell population, two blots were obtained and independently hybridized with the mouse CSN2 and rabbit CSN1 probes (**right panels**). No signal is observed for rabbit RNA hybridized with the mouse CSN2 probe. For the D2 cell population, one blot has been sequentially hybridized with the rabbit CSN1 probe and then with the mouse CSN2 probe (**left-hand panels**). A signal is then observed on rabbit RNA after hybridization with the mouse CSN2 probe (**left-hand middle panel**). This signal is therefore considered as residual from the first hybridization with the rabbit CSN1 probe. The blots hybridized with the rabbit CSN1 probe were exposed to Phosphor screen for 72 h (D2) or 4 h (D8), whereas the blots hybridized with the mouse CSN2 probe were exposed for 48 h. Gels were stained with ethidium bromide (**lower panel**) to check for the quality of RNA and loading of the gel.

copies, respectively, confirming the evaluation obtained by ImageQuant analysis of Southern blots.

Interestingly, the various expression levels were not related to the copy number of the transfected rabbit *CSN1* gene (Table I).

In CSN1 Expressing Cells, the Transfected Gene Remains Attached to the Nuclear Matrix on Nuclear Halos

Recent publications have shown that several interrelated mechanisms contribute to the

TABLE I. Evaluation of Rabbit CSN1 Expression in HC11 Cells

Origin of RNA	Transgene copy number	Rabbit CSN1/ cyclophilin	Mouse CSN2/ cyclophilin	Cyclophilin/ ng total RNA
Clone D2	104	19	63	74
Clone D8	46	171	2	61
Clone C9	60	30	174	46
Clone C5	69	83.5	0.06	52

In mouse HC11 mammary cells transfected with BAC containing the rabbit *CSN1* gene, transgene copy number was estimated by real time PCR, and levels of rabbit CSN1 and/or mouse CSN2 mRNA were evaluated using real time RT-PCR relative to mouse cyclophilin mRNA levels (/1,000).

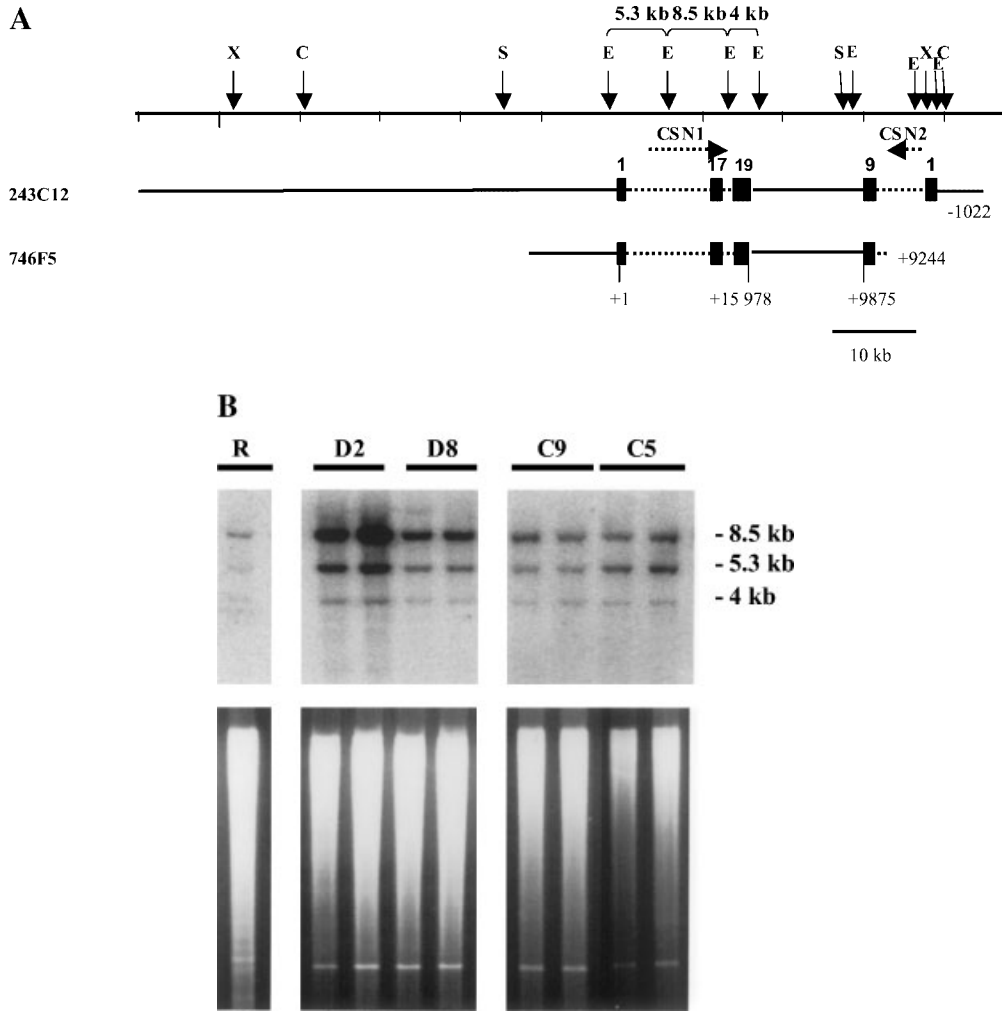

Fig. 2. Analysis of rabbit *CSN1* gene copy number, by Southern blot, in D2, D8, C5, and C9 clones. **A**: Schematic representation of BAC 243C12 or 746F5 DNA which was transfected in clones D2, D8 and C5, C9, respectively. Some characteristic restriction sites are indicated: C: *Cla* I, E: *Eco* RI, S: *Sma* I, X: *Xho* I. Only the first and last exons are depicted as gray boxes, the other exons and introns have been figured as dotted lines. **B**: Genomic DNA (10 μg) from D2, D8, C5, and C9 clones or rabbit liver (R) was digested by *Eco* R1, analyzed by electrophoresis on a 1% agarose gel, transferred to a Nylon membrane and hybridized with a rabbit CSN1 full length cDNA probe (**upper panel**). Ethidium bromide staining of the gel is shown on the **lower panel**.

regulation of gene expression, including the nuclear positioning of genes and their attachment to a nuclear matrix [Bode et al., 2003]. With respect to the clones described above, we therefore analyzed the degree of attachment of the rabbit *CSNS1* gene to the nuclear matrix. We applied the technique previously described by Gerdes et al. [1994], adapted to our in situ hybridization procedure, in order to visualize DNA looping out of the nuclear structure (nuclear halos) or remaining within the nucleus after the high salt treatment of nuclei. The quality of the prepared halos was verified using combined lamin A/B immunolabeling and Hoechst staining (Fig. 3).

In D8 and C5 cells expressing high level of the rabbit *CSN1* gene, the transgene appeared to be strongly associated to the nuclear matrix, since in most cases it remained within the nucleus after expansion of the chromatin loops (Fig. 4, right-hand panel). In a few cases, the signal was at the nuclear periphery but was never clearly in the extended portion of the halo (Fig. 4, right-hand panel). On the contrary, in cells expressing low level of the rabbit *CSNS1* gene (D2 and C9), most of the hybridization signal could be found in the extended portion of the halo (Fig. 4, left-hand panel). Similar results were obtained in several independent experiments, as summarized in Table II. Taken together, these

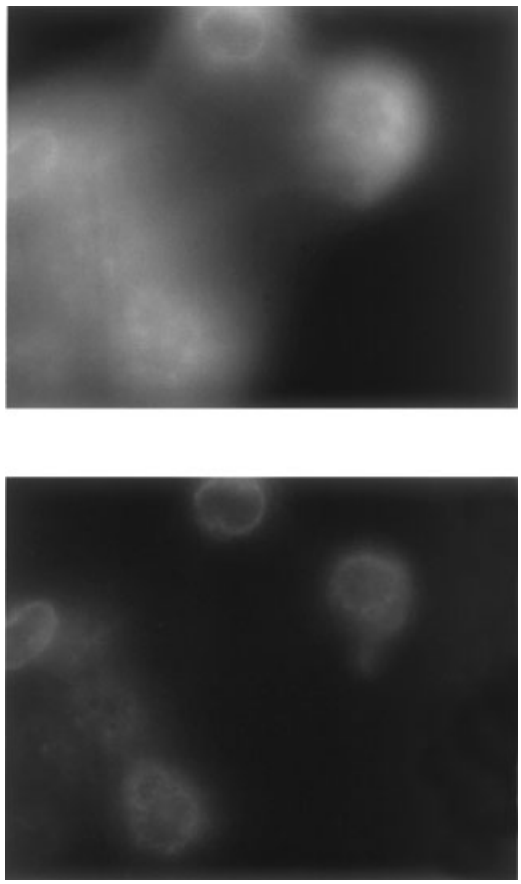

Fig. 3. Evidence for DNA extrusion out of the nuclear area after halo preparation. DNA was stained by Hoechst (**upper panel**) and the nuclear membrane was immunolabeled with a lamin A/C antibody (**lower panel**).

experiments showed that in the cell populations expressing high levels of the transgene (D8 and C5), the proportion of nuclei in which the transgene remained within the nucleus after high salt treatment (91% and 86%, respectively) is significantly different from that observed in cell populations expressing low levels of the transgene (8% and 23% for D2 and C9, respectively) (Student's test, $P < 0.0025$). Conversely, the number of nuclei in which the transgene is found in DNA loops after halo formation is also different for the two cell populations. These experiments allowed us to detect a positive relationship between the degree of attachment of the *CSNS1* gene to the nuclear matrix and its level of expression in the cell population studied.

However, for several nuclei we did not observe a single hybridization signal but several signals. We therefore decided to check for the presence of hybridization signals on metaphase spreads.

Multiple Transfected *CSN1* Gene Integration Sites Are Frequent in HC11 Transfected Cells

Metaphase spreads were prepared for each of the four clones described above, and were hybridized in situ with the labeled rabbit CSN1 BAC probe (243C12). Representative images obtained with each clone are shown in Figure 5 and the results obtained from several independent metaphase spreads are listed in Table III. The number of chromosomes was estimated on more than 21 metaphase spreads for each transfected cell population as well as on original HC11 cells which had not been transfected. In transfected cells, these numbers ranged from 68 to 84, with a mean value of 78.5 chromosomes per metaphase (including 5–6 punctiform chromosomes). There was no significant difference between transfected and original HC11 cells, where the number of chromosomes ranged from 75 to 85, with a mean value of 79.7.

Of the 28 metaphase spreads analyzed from D2 cells, 36% were found to display a single integration site for the rabbit *CSNS1* gene on a small or middle-sized chromosome. Two integration sites were found in 46% of metaphase spreads, in the form of a very bright signal on a small chromosome and a weak signal on a middle-sized chromosome (Fig. 5). As for the other metaphases (18%), 3 or 4 signals were found on different chromosomes, the presence of which in some cases could be explained by the duplication and separation of chromosomes. These results suggest that this clone may be a mixture of at least two cell populations.

In most of the D8 cells analyzed, only one integration site was found. In the majority of these, a middle-sized chromosome was labeled (98% of cases) with either a hybridization spot (62%) or a signal throughout the arm (38%), depending on the condensation stage of the chromosome (Fig. 5). In the remainder of metaphases (2%), two small chromosomes were labeled one with a small sharp spot and the other with a stronger broad signal. These results suggest that this clone may be a mixture of at least two cell populations.

In C5 cells, a single integration site on a long chromosome was observed in 73% of metaphases (Fig. 5). In the other 27%, two integration sites were detected, either on two small or

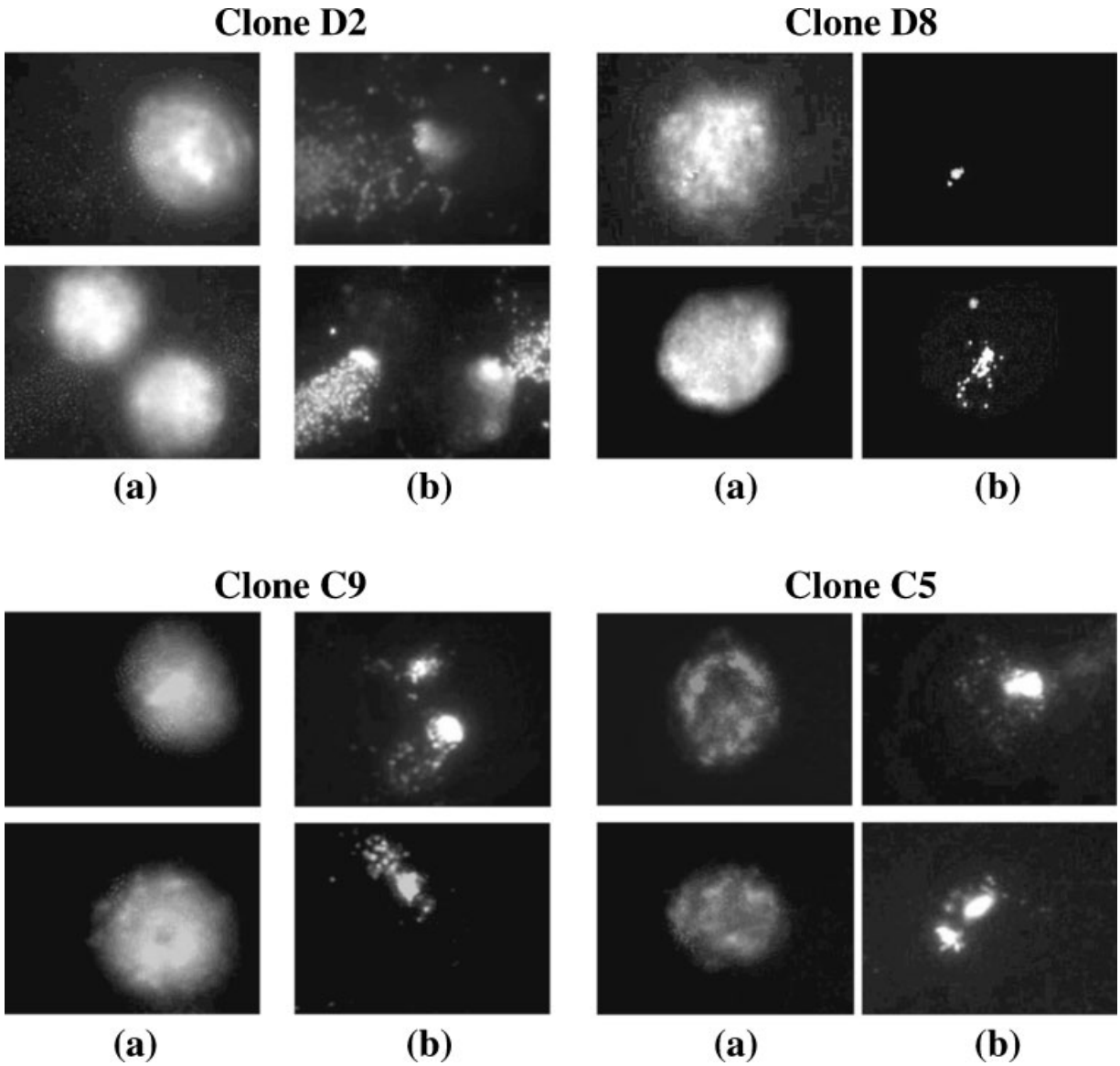

Fig. 4. Attachment of the rabbit *CSN1* gene to the nuclear matrix varies as a function of its level of expression in the cell population. Nuclear halos obtained for cells from clones D2, D8, C5, and C9 were hybridized in situ with a rabbit CSN1 genomic probe (b columns). DNA was counterstained with Hoechst and the same fields as in b are shown (a columns). Representative results of two preparations are shown for each clone.

two long chromosomes, indicating a mixture of two cell populations instead of a pure clone.

In the case of C9 cells, the results were very homogeneous with two long chromosomes being labeled in all cases (Fig. 5).

In conclusion, these results suggest that after one cycle of selection of isolated transfected cells, the cell population remained largely heterogeneous. Independently of a low efficiency of selection, this heterogeneity might also result from the genomic instability of cells transfected with a large number of BAC copies. Since the HC11 cells are known to loose the ability to express *CSN2* gene after several passages, we decided not to pursue the cloning procedure by several rounds of selection.

More interestingly, the results show that the most frequent event occurring under our conditions of stable transfection was a double integration of the transfected DNA. When chromosomes were sufficiently long to distinguish centromeres and chromosome arms, the integration sites were not located close to the centromeric regions (Fig. 5).

TABLE II. Distribution of Hybridization Signals With the Rabbit CSN1 BAC Probe, After the Treatment of Nuclei to Generate Halos

Clone	Loop (% of total)	Nuclear remnant (% of total)	Total
D2	35 (92)	3 (8)	38
D8	3 (9)	30 (91)	33
C9	10 (77)	3 (23)	13
C5	1 (14)	6 (86)	7

For each clone, the number of nuclei in which a signal was detected in extra-nuclear loops or in nuclear remnant DNA, following hybridization with the rabbit CSN1BAC probe, is indicated. The total number of nuclei for which halos were clearly formed is shown in the last column.

DISCUSSION

During this study, we examined the chromosomal content of mammary HC11 cells for the first time. The number of chromosomes per metaphase spread was variable, with a mean value of 78.5. Variations were probably due in part to spreading artefacts, resulting in overlapped or masked chromosomes which were counted as a single chromosome. For this reason, the mean value of 78.5 was probably underestimated but remained clearly higher than the $2n = 40$ chromosome number of a normal mouse karyotype [Nesbitt and Francke, 1973]. Normal mouse chromosomes are all acrocentric. Our observations of HC11 metaphase spreads revealed that most of the chromosomes

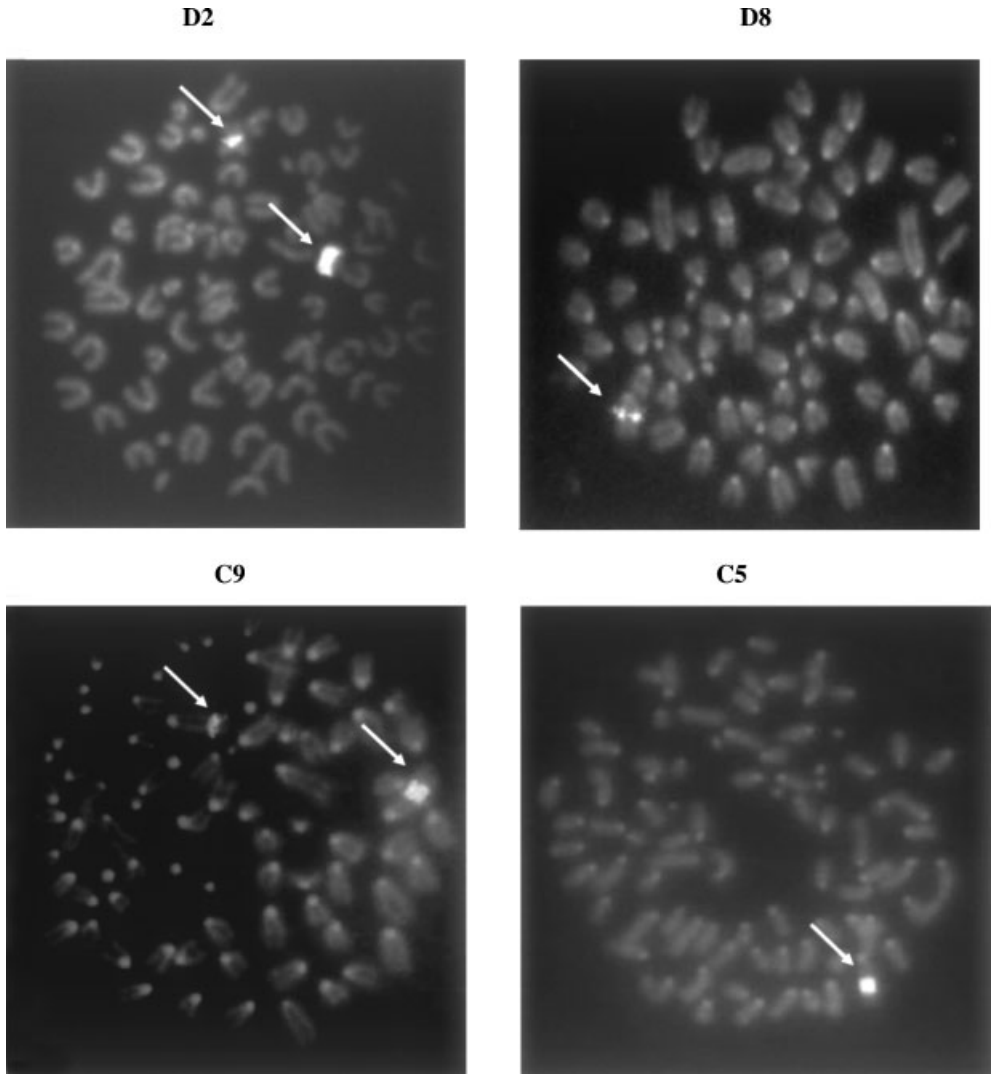

Fig. 5. Analysis of the rabbit *CSN1* gene integration site on chromosome preparations from HC11 transfected cells. The metaphase spreads were hybridized with a rabbit CSN1 probe (bright signals indicated by arrows); DNA was counterstained with Hoechst.

TABLE III. Number of Hybridization Signals Per Metaphase

Clone	1 spot	2 spots	3, 4, 6 spots	Total
D2	10	13	5	28
D8	56	1	0	57
C9	0	12	0	12
C5	16	6	0	22

For each clone, the number of metaphases in which one or two signals were observed is indicated in the second and third columns, respectively. In clone D2, a few metaphases exhibited 3, 4, or 6 spots, as indicated in the fourth column. The total number of metaphases studied is shown in the last column.

were also acrocentric, except in the D8 clone where a metacentric chromosome was observed in several metaphase spreads. This suggests that the HC11 cells had undergone a genome duplication or tetraploidization event followed by a few secondary chromosome changes such as robertsonian translocations during culture. Such events would explain the chromosome number in HC11 cells close to $4n = 80$. Further investigations of banded karyotypes are necessary to describe their karyotype more precisely and compare them with the normal mouse karyotype. However, these preliminary data indicate that mammary HC11 cells have not maintained a normal mouse karyotype. This may explain why these cells do not fully reflect the physiological functioning of epithelial cells in the mammary gland and, for example, do not express all milk protein genes.

The results described above show that the level of rabbit CSN1 expression in transfected HC11 cells varies from clone to clone, a variation which is not related to the length of the CSN1 flanking region (BAC 243C12 or 746F5), but may depend on the integration sites on chromosomes. It is worth noting that "silencing" of the introduced gene could occur even when the transfected DNA was not located in the centromere or telomeric regions. In each clone which was analyzed, the number of integrated copies was very high and copies were all located at one or two sites. Previous reports have shown that in transgenic mice, transgenes integrated at a high copy number tend to be methylated and not expressed [Mehtali et al., 1990]. This might explain why we only have a few expressing clones in our experiments. However, a recent paper shows that in transfected cells, a transgene integrated at 200 copies can be expressed [Janicki et al., 2004]. Similarly to the data reported by Janicki et al. [2004], our experiments show that expression of the transfected gene is possible even if it is integrated at a high copy number, possibly because cells were kept only for a limited number of cell cycles. In the case of an integration site permitting a high expression level of the transfected gene and when the transgene is present at a high copy number, its regulatory regions may trap positive transcription factors such as activated Stat5 and this may induce a down regulation of the endogenous *CSN2* gene. In the absence of prolactin, a similar phenomenon may induce a competition for negative regulatory factors and an abnormal expression of the transfected gene.

Alternatively, the expression levels may depend on the methylation state of the transfected gene. Our preliminary results indicate that methylation-sensitive restriction sites in milk protein genes are less methylated in the mammary gland than in the liver (data not shown). In the four clones studied above, we therefore studied the methylation state of one of those sites, a *Sal* I site located in intron 15 of the rabbit *CSN1* gene. In all four clones, this site appeared to be uncut and was therefore presumed to be methylated. However, it should be kept in mind that these clones harbored between 46 and 104 copies of the *CSN1* gene and that some of the copies could be not methylated. The only possible conclusion is therefore that the expression levels were not clearly correlated to the methylation state of the transfected gene. Since the methylation status may be related to the chromatin structure surrounding the transgene, it would be interesting to analyze the chromatin environment of the transgene and to see whether it is found in heterochromatin protein 1 (HP1) rich regions.

The interesting results of this study is that, in cell populations expressing high levels of the rabbit CSN1, in most cases (86%–91%) the transfected genes remained within the nucleus under hypertonic conditions, whereas in cell populations expressing low levels of the transfected gene, the transfected genes looped out from the nucleus in 77%–92% of observed nuclei. Even though all the cell populations studied were not of clonal origin but only enriched in a dominant population, which in three cases out of four represent 73%–100% of cells, this finding, observed in 77%–92% of nuclei, cannot be accounted for by a minor population of cells. It may therefore indicate that in highly expressing cells, genes are more tightly attached to the nuclear structure than in low expres-

sing cells. It would now be interesting to determine whether this possible attachment is regulated by lactogenic hormones. However, in the clones we studied, and as already described for some constructs transfected in HC11 cells [Doppler et al., 1991; Pauloin et al., 2002], the expressing or non-expressing status of the transfected gene is not regulated by prolactin. Further studies should therefore be planned in another, more physiological model. The native (not transfected) HC11 cells in which endogenous *CSN2* gene expression is regulated by lactogenic hormones might be used. However, as the genome of this cell line is duplicated, we expect the cells to carry four *CSN2* genes, and any study will first have to check if the four *CSN2* genes are expressed.

ACKNOWLEDGMENTS

The authors thank Claire Rogel-Gaillard for advices in using BAC clones and M. Bagher Torbati for his help in methylation studies.

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