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Recent data on the structure of rabbit milk protein genes and on the mechanism of the hormonal control of their expression

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Summary. Mammary explants or isolated mammary cells from rabbit have been cultured in the presence of insulin, prolactin and cortisol alone or in combination. The cellular content in α_{s1}-casein, β-casein and whey acidic protein (WAP) mRNA have been evaluated using the corresponding cDNA as probes. In all cases α_{s1}-casein mRNA was the most abundant and WAP mRNA the least abundant mRNA. The three genes showed essentially similar dependency towards hormones. Prolactin stimulated mRNA accumulation and insulin and cortisol amplified this stimulation. The induction by prolactin was rapid whereas stimulation by insulin was slower. Fragments of rabbit genomic DNA inserted in λ phage and containing α_{s1}-casein, β-casein and WAP genes have been cloned. The primary sequence around the CAP site of the three genes has been established. A comparison of the sequences located upstream from the CAP site shows several striking homologies with the corresponding genes from cow, rat and guinea-pig. This suggests that these sequences participate in the transcriptional control of the genes by hormones. The mechanism involved in the transduction of the prolactin message to milk protein genes in unknown. Using mammary explants in culture, several classical mechanisms of transduction have been examined. Phorbol ester, phorbol -12, 13-dibutyrate (PdiBu) inhibited prolactin action. However, another tumor promoter, 12-0-tetradecanoyl phorbol -13-acetate (TPA), did not alter prolactin action. Kinase C inhibitor H7 did not prevent prolactin action and did not overcome the inhibition by PdiBu. Kinase C is therefore not essential for the transduction of the prolactin message to milk protein gene. Neomycin, which inhibits phosphatidylinositol hydrolysis by phosphorylase C, prevented prolactin action, whereas other inhibitors of phosphatidylinositol metabolism remained uneffective. Degradation of phosphatidylinositol is therefore likely not an essential step of prolactin action on milk protein genes. Inhibitors of tyrosine kinase and phosphatase exhibited a poor capacity to modify the prolactin response. Hence, transduction mechanisms using tyrosine kinase activity likely cannot account for prolactin action.

Introduction.

Milk protein synthesis is under the control of several hormones. In the rabbit, freshly explanted mammary tissue is sensitive to prolactin alone whereas insulin

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and glucocorticoids greatly enhance in an independant manner the accumulation of β-casein mRNA (Houdebine *et al.,* 1985). Progesterone *in vivo* (Teyssot and Houdebine, 1980) and *in vitro* (Jahn *et al.,* unpublished results) inhibits prolactin action. Prolactin receptor has been isolated and characterized. Polyclonal and some monoclonal antibodies raised against the receptor can mimic prolactin actions (Djiane *et al.,* 1981, 1985). The hormone is therefore not necessary beyond its receptor, and a mediator of unknown nature must be generated at the membrane level (Houdebine *et al.,* 1985). In order to elucidate the essential steps of the mechanism of prolactin action on milk protein gene expression, a study of the hormonal receptor at the molecular level and of several prolactin target genes have been undertaken. The data reported in the present paper are related to the structure and the hormonal control of three rabbit milk protein genes. Experiments carried out in many different biological systems have shown that the essential regulatory elements for transcription are located in the upstream region of the genes (Maniatis *et al.,* 1987). For that reason, the structure of the regions upstream from the CAP site of rabbit α_{s1}—casein, β-casein and WAP genes have been determined and compared to homologous genes of other species. Several experiments aimed at determining the possible transduction mechanism of the prolactin message to milk protein genes are also reported.

Results.

I. Hormonal control of the expression of rabbit milk protein genes.

Previous studies have shown that in the rabbit, β-casein gene expression is triggered by prolactin in cultured mammary explants and that this action is amplified markedly by insulin and cortisol (Houdebine *et al.,* 1985). Results of figure 1 indicate that the same is true for two other rabbit milk protein genes, α_{s1}-casein and whey acidic protein (WAP) genes. In all cases, *in vivo* and *in vitro,* α_{s1}-casein was the most and WAP the least abundant of the three mRNAs. Interestingly, isolated rabbit epithelial mammary cells cultured on floating collagen exhibited essentially a similar sensitivity towards the three hormones (fig. 2). In this respect, rabbit appears different from mouse in which WAP gene is expressed in cultured explants but no more in isolated cells (Lee *et al.,* 1985).

Mammary explants cultured for two days in the presence of insulin and cortisol kept an excellent sensitivity towards prolactin which triggered a rapid accumulation of β-casein mRNA (fig. 3). As opposed, tissue cultured for two days in the absence of hormone had lost a large part of its capacity to respond to the prolactin stimulation (not shown). The mammary explants cultured for two days in the presence of only prolactin could be stimulated by insulin for the accumulation of β-casein but this stimulation took place relatively slowly (fig. 4). Insulin is known to stimulate or inhibit specific gene expression and its action is slow in some cases (Flaim *et al.,* 1985) and rapid in others (Lee *et al.,* 1986). Insulin action on casein gene belongs rather to the first category.

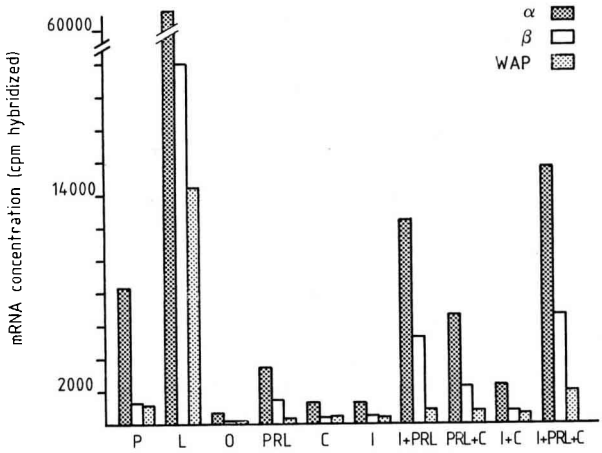

FIG. 1. A. — *Effect of hormones on the induction of α_{s1}-casein, β-casein and WAP mRNA in mammary explants.* Rabbit mammary fragments explanted at day 13 of pregnancy were cultured for 48 hours in the presence of insulin (I : 5 µg/ml), cortisol (c : 500 ng/ml) and prolactin (PRL : 1 µg/ml) as mentioned on the figure. Three cultures were performed independently. Total RNA was extracted from explants in each case and equal amounts of RNA of each hormonal treatment resulting from the three cultures were mixed. RNA (10 µg) was bound to nitrocellulose filters and hybridized separately with the three cDNA probes (10^8 cpm/µg DNA in each case). Results which are the means of triplicates are expressed as cpm of the cDNA probe specifically hybridized to filters. P and L designate pregnancy and lactation respectively. Hybridization was then carried out with RNA isolated from fresh tissue.

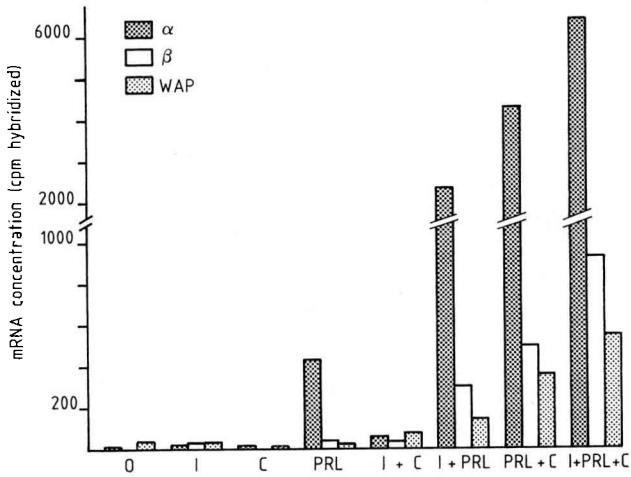

FIG. 2. — *Effect of hormones on the induction of α_{s1}-casein, β-casein and WAP gene in isolated cultured mammary cells.* Cells were cultured on floating collagen as previously described (Servely *et al.,* 1987). About 5 × 10^6 cells were present in each dish. The whole RNA was bound to nitrocellulose filters and hybridized to labelled cDNA probes (the three probes had not the same specific activity). Results which are the mean of triplicates are expressed as cpm of the probe specifically hybridized to filters.

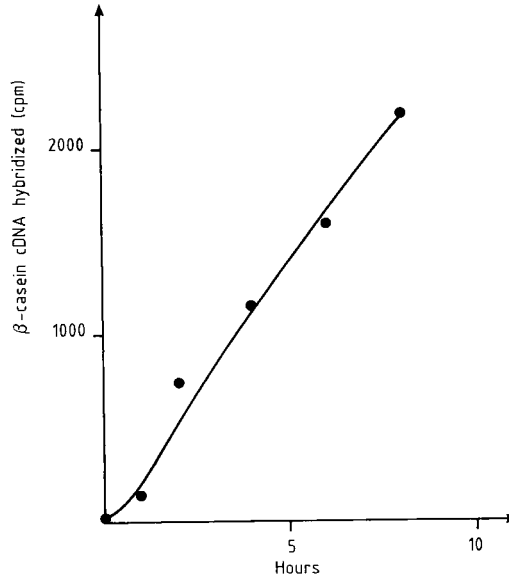

FIG. 3. — *Kinetic of induction of β-casein mRNA accumulation by prolactin.* Mammary explants from mid-pregnant rabbit cultured for two days in the presence of insulin (5 μg/ml) and cortisol (500 ng/ml). Prolactin (1 μg/ml) was then added to the culture medium. Culture of explants was stopped at different times after the addition of prolactin. The presence of β-casein mRNA was evaluated with a specific cDNA probe as depicted in fig. 1.

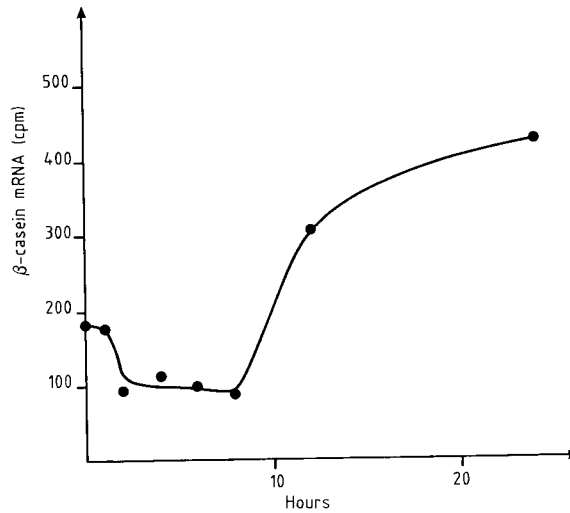

FIG. 4. — *Kinetic of induction of β-casein mRNA accumulation by insulin.* Mammary explants from mid-pregnant rabbit cultured for two days in the presence of prolactin alone (100 ng/ml). Insulin (5 μg/ml) was then added and β-casein mRNA was measured at different times after the addition of insulin as depicted in fig. 1.

II. Determination of the structure of the upstream regions of three rabbit milk protein genes.

Experiments carried out several years ago in different laboratories have shown that the expression of milk protein genes is regulated at the transcriptional level by prolactin, insulin and glucocorticoids and that the half-life of the corresponding mRNA is greatly enhanced by prolactin and glucocorticoids (Houdebine *et al.*, 1978 ; Guyette *et al.*, 1979 ; Ganguly *et al.*, 1979 ; Teyssot and Houdebine, 1980 ; Chomczynski *et al.*, 1984, 1986). Signals controlling milk protein gene transcription are expected to be located in the upstream region of their CAP sites whereas other signals controlling mRNA stability are expected to be present in the mature mRNA. Recent experiments using mouse WAP gene regulatory sequences fused to ras oncogene and to plasminogen activator gene have shown that the WAP gene sequences are able to selectively direct the expression of the foreign genes in the mammary gland, although at a limited rate (Andres *et al.*, 1987 ; Gordon *et al.*, 1987). On the contrary, the whole sheep β-lactoglobulin gene was expressed with high efficiency in the mammary gland of transgenic lactating mouse (Simons *et al.*, 1987). To address this problem, the cDNAs for rabbit α_{s1}-casein, β-casein and WAP have been cloned (Suard *et al.*, 1982 ; our laboratory, unpublished results) and sequenced (results to be published elsewhere). These cDNAs have been used as probes to select λ phages, harbouring the corresponding genomic DNA, from a genomic library prepared in T. Maniatis' laboratory (Maniatis, 1978).

1) *Characterization of the clones.* — One clone isolated, using the α_{s1}-casein cDNA as probe, harboured a 12 Kb fragment of genomic DNA which contains the 5'half of the corresponding mRNA (450 bp) and 10 Kb upstream from the CAP site.

The clone having β-casein sequence contained, in 11 Kb, the entire gene encoding this milk protein mRNA (1 200 bp). This clone also contained 320 bp in the upstream region of the CAP site.

One of the clones harbouring the WAP cDNA sequence contained about half of the WAP mRNA sequence (260 bp) and 17 Kb in the upstream region of the CAP site.

Classical genetic studies (Grosclaude *et al.*, 1978) and kinetics of hybridization of cDNA with whole genomic DNA (Houdebine, 1977) have shown that essentially one copy of each casein gene is present per haploid genome. This fact was later confirmed for rat WAP, α_{s1}-casein and β-casein genes using southern blotting (Campbell et Rosen, 1984 ; Jones *et al.*, 1985, Yu-Lee *et al.*, 1986). The same was observed for rabbit β-casein gene (unpublished result).

In the three genomic fragments we have isolated and described above, sequences of the corresponding cDNA were found. Therefore, these isolated genomic DNAs are fragments of the single α-casein, β-casein and WAP genes expressed in the mammary gland.

2) *Sequence of the upstream regions of the three genes.* — Results of figures 5, 6 and 7 indicate that the three genes have a TATA box located at the expected position, *i.e.* between -24 and -30 bp from their CAP site. β-casein gene

A.

```
                    gaattcttagaatttaaataaacctattggttgatctgaaacc  -61
                    ==========2==========                ====

acaaaattagcattttactgatcactgggtttaaatacttgtgaagcaaagagatcgacc   -1
=====1=============

   ATCGCCTAGATCATCAACCCAACTTGCCTCTTTTCAGTCGAGTTTAAG   gtattg   54

gtgatcaaatcatacgatttcacactgtctaatgtctttcttctgctttttattaagatg  114

tatttgtaacttttctatgttggaaaatgtgatacctttttaagatatactggtagcttt  174

aaaatgcattttcaaaattttaatactatctcagagctttaggtaagaaatgtatatggc  234

attataaagaccaaatcatttttgcttaatgtgtaactatatctcatacacttatcactt  294

atctttcaatagaaacttagaataaggtaatttgaattcttttagcccaattctatgaaa  354

ttatttgtgaaaaataatttactatctttgcaattgctatagcaaggcttgttttggtag  414

aatatttcaagactcagttttattccatatttgtggctattaaaattaattttataccat  474

gtaaattaaaaaacaccagttatctatagataaatattttataaaggtgattaaatttat  534

atatttggaaaggacaaaaatgtggataaacagtaagttgaattttgatttagaacaggc  594

tttctttacttttgctcttaggatctacataaactgaagcaatacactattagtttgctg  654

acaaattaaaaatgtagcctaatgtttttttgatttccctgaaaatcattttcgatggta  714

catgtaaatgtcagtatttctgaggttcttccaatttgatggaactgcagtcatcaaata  774

tgatatgatatatgaatattatgcctctttacatttcatccatcttcgatatattctata  834

gctgtaaatatttcaatgctgcaaaaacgatgatgtctggattgcctttaaaaaataact  894

gatgcgacggttaatag
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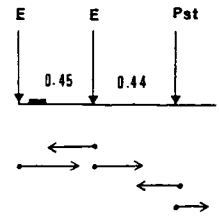
B.



911

FIG. 5. A. — *Upstream sequence of the rabbit α_{s1}-casein gene and the first intron.* Sequence analysis was carried out according to the method of SANGER *et al.* (1977) using [α-^{35} s]-dATP as marker and 0.2 mm thick gels run in an LKB apparatus. Underlined sequences are detailed in fig. 8, 9, 10 and 11.
Exon sequences are shown in upper case letters.
B. — *Strategy of sequencing.* Exons are represented by solid boxes.

```
                                                  tcatgcctccctagacccaa -300

aagaaacctaatagtgctcacattagtatcttggagactaggctggagcaccactcttct -240

ccagccattgtgtttattattgggcaattcatttcctggacacatttcctttataggcct -180

                          consensus Pg
atgaattactgccttgtctcaatgctccccagaatttctggggaaagataatgagtagaa -120
                          _ _ _ _ _ _ _      ==================3===

atcattttctaatcatatggagtt cttggaattgaaactcaaaatcgatttttttttc  -60
==========          ========2========

aaaccacaaaattagcatgtcattaaatgcagtatataagcatccccaaaagcagagaac   -1
==========1==============

   ATCATCCACCCAGCTTCATTTTCACTTCTTGTCCTCCACCTTGGAATAAAG   gta   54

ag                                                              56
```

FIG. 6. — *Upstream sequence of the rabbit β-casein gene and the first exon.* (See legend of fig. 5).

showed a typical TATA consensus sequence (Breathnach et Chambon, 1981) whereas both α_{s1}-casein and WAP had the sequence in a modified form. Interestingly, this somewhat unusual TTTAAAT sequence has been found in most of the milk protein genes so far studied (Campbell et Rosen, 1984 ; Yu-Lee *et al.*, 1986). Moreover, an additional TATA box located 53 bp upstream from the first TATA box was found in rabbit α_{s1}-casein gene. It is striking that this fact was also mentioned for bovine α_{s1}-casein gene which gives rise to the formation of a minor α_{s1}-casein mRNA starting from position -35 bp (Yu-Lee *et al.*, 1986). In addition, the rabbit β-casein gene contains the ATTAAATGCA sequence upstream from the classical TATA box. This sequence is reminescent of that found upstream from the WAP gene TTTAAATGCA. This sequence in the β-casein gene might be, as for α_{s1}-casein gene, a second functional TATA box.

The CAAT box present in many other eucaryotic genes (Breathnach and Chambon, 1981) could not be determined in any of the three genes. Glucocorticoid receptors are known to stimulate their target genes by interacting with a sequence found on the upstream region of these genes (Hutchinson *et al.*, 1986). The nonanucleotide consensus sequence which binds glucocorticoid receptor has not been found yet in any of the three rabbit milk protein genes. However, the hexanucleotide sequence AGGAAG (Hall *et al.*, 1987) was repeated five times (with two of them in the opposite direction) in the upstream part of the rabbit WAP gene. These sequences possibly take part in the regulation of WAP gene transcription. However, experiments not depicted here have shown that, although glucocorticoids exert a strong effect on β-casein mRNA accumulation (fig. 1), their stimulation is rather slow, suggesting that their action might not be mediated through direct binding of glucocorticoid receptor to milk protein genes.

In rabbit β-casein gene, at position -146 bp, a sequence of 14 nucleotides shares a 86 % homology with the chicken sequence which has been shown to

agatcttgtgctcgct-18Ø1

cgctctctcgctctctctctctcttctgtctctctggaactttgcctttcaaataaa-1741

taaataattttttaaaagactactgttttgtttttttatttacttaaagcagagtaac-1681

agagaaagaaatacattccgtttgctggttcactccccaaatggccgctagatccagggc-1621

taggccaggctgaagccagaacccctacctgggtctcccacgtgagtgacaggggcccaa-1561

gcacctgggccaaccacctctgctttcccagggacattggcagggagatgggtcaggagc-15Ø1

agagcagccagaactcaggctgccctccaatctgagacatcagctttgcaagtggtagct-1441

taacccacgtgtcacccagccccaagattcatgttaatgataggaaattttaattttatt-1381

tgctcagattgaaacattataaaggcaccacaataagcagagtccagagatgagagaaaa-1321

acaaaaaataaaataaaaaaatcttgtatttcggttcctttgcaggcactttcttccctt-1261

tgtggaacaaggagcccaaaaaccgcagcagggggcccagtggagatgggagatgcctgg-12Ø1

gaagaacaccctgggaggagctccgggaggcgcaggaggagggggttcctgacggggtca-1141

gctctggcctcgggcccagcaccccagtgagaaggatgggagccgcccagcccagcctgg-1Ø81

ctcgggcaggaaggggcaggcccaaccacagcccctctgctccttcgcagggagcggaac-1Ø21

agcccacggaagcatctttcggacttagagccgtgaacctcgccacgccgtgtccagccc -961

actgtctgagagccctcactggccagtccaggcccaggcccaggactctgtgggcagctg -9Ø1

cagggctggaacagagttacccgagcctggggctgcgaggggtgcctttgtggaacccac -841

aaaggacgctttgtggaaggacatttggggctggagcctccccacggcacagcctgaggc -781

ccaggaagctgcgaggagctctgtgcctgaggccggagcagggtcgctggctggacaggg -721

ctgtggcccccagccatcctgccctggggtctccgcagtccccatggccccttccctgtc -661

```
tgggtcctgggggggcgggtgcaggaactacacggccagcagcacatccgcccctgccct -601

gtggcacctgctcccctggcacagggcacaggagggccttccgagaagagaccttctgtc -541

ccctcgcccctccacagtcggcaagcctgcactggggtccccagggcaggggcccaggct -481

ctgcagtccgcttctcctgtcccctcgcccctccacaggtggcaagcagcacattcttgc -421

ttacagagtccagaaaaccacacacacacacacacacacacacacacacacacacacaca -361

aaaaaaaacacttgccgacgagacagcccgcacttggtacccgcctcccatgctgcttct -301

cccggctctgagccgtgggtacaaccctcgggggggggggaggatttctctcccccaccc -241

ccagtcttcctagcagatgtgcatcccggccaacatggagggaaatggacaaccttgccg -181
                                     ═══════3═══════

gggactttttttctttcatttgaaaccatgaccgcagccgttcctccaacctggcctga -121

cctctccacgtgtccaaggaggaagcccccctggcccagttgaggcctcgccaacctggca  -61

cccctccaggctcctcctcctgctccaacctttaaatgcatcccggggccccagaacacc   -1

   ATCCGACACCTGCCTGCTGCCCACCACCAGCCTACCACCTGCCACC   ATG CGC    52
                                                    Met Arg

TGT CTC ATC AGC CTG GCC CTC GGC CTG CTC GCC CTG GAG GCG GCC    97
Cys Leu Ile Ser Leu Ala Leu Gly Leu Leu Ala Leu Glu Ala Ala

CTC GCT CTG GCC CCC AA...                                     114
Leu Ala Leu Ala Pro
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B.

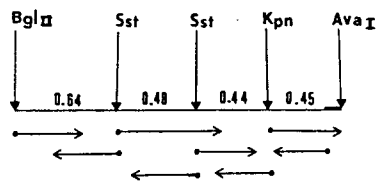

FIG. 7. A. — *Upstream sequence of the rabbit WAP gene and the first exon.* (See legend of fig. 5).
B. — *Strategy of sequencing.*

interact with progesterone receptor in chicken ovalbumin gene (Compton *et al.*, 1983). However, the functional significance of this sequence is quite uncertain. Indeed, ovalbumin gene is stimulated by progesterone whereas milk protein genes are inhibited by this steroid. Further, recent experiments have demonstrated that the DNA sequence of the rabbit uteroglobin gene, which binds progesterone receptor, is quite different from that proposed for chicken ovalbumin gene (Bailly *et al.*, 1986). This rabbit uteroglobin sequence was not found in the upstream regions of the rabbit milk protein genes so far examined.

3) *Sequence homologies of the different genes.* — Apart from the TATA box, a short sequence of seven nucleotides was similar downstream from the TATA sequence in the WAP and the β-casein, but not in the α_{s1}-casein gene (fig. 8).

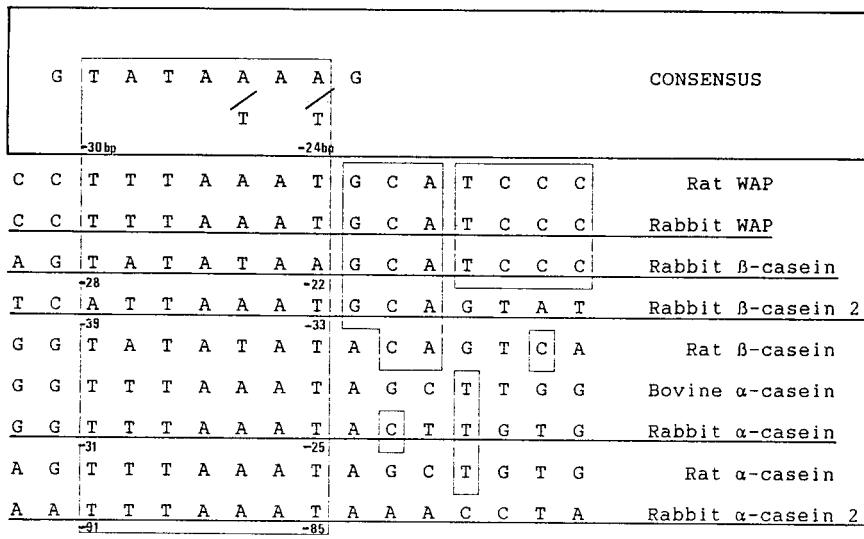

FIG. 8. — *Sequence homology between milk protein genes from various species in the region of the TATA box.* Comparison of sequences was carried out according to the method proposed by Kanehisha (1984).

Recently, it has been proposed that a conserved sequence might be present in the regulatory regions of all the milk protein genes (Hall *et al.*, 1987). This sequence has been found in human α-lactalbumin gene and in several casein genes from guinea-pig and rat (Qasba et Safaya, 1984). An analogous sequence was also found in the upstream region of the rabbit β-casein gene (fig. 9). The same sequence, or at least a part of it, was also present in the rabbit WAP gene (fig. 9). Sequencing in progress will soon reveal if this consensus sequence is also present in the rabbit α_{s1}-casein gene.

```
G G A G G G A A A - T G G A C A                                  Rabbit WAP
                                 -199
R G A A G R A A A N T G G A C A G A A A - T C A A C G T T T C T A  Consensus
G G A A A G A T A A T G A G T A G A A A - T C A T - - T T T C T A  Rabbit
                                                                   ß-casein
G A A A A A A A A - T A G A A A G A A A - T C A T - - T T T C T A  Rat ß-casein
-139bp                                                        -110bp
```

FIG. 9. — *Sequence homology of a region of the three rabbit genes with the consensus sequence defined by* Hall *et al.* (1987).

```
G A A A C C A C A A A A T T A G C A T T T T A C T A A   Bovine α-caseine
G A A A C C A C A A A A T T A G C A T T T T A C T G A   Rabbit α-casein
A A A A C C A C A A A A T T A G C A T T T C A C T G C   Rat α-casein
C A A A C C A C A A A A T T A G C A T G T C A T T A A   Rabbit ß-casein
C G A A C C A C A A A - T T A G C A T G T C A T T A A   Rat ß-casein
G A A A C C A C A G A A T T A G C A T - T A A A T G C   Rat γ-casein
-60 bp                                          -40 bp
```

FIG. 10. — *Sequence homology between milk protein genes from various species in the upstream region of the TATA box.*

A highly conserved stretch of DNA, located in the -60 bp -40 bp region of several rat (Jones *et al.,* 1985 ; Yu-Lee and Rosen, 1983 ; Yu-Lee *et al.,* 1986) and bovine casein genes was also found in rabbit α and β-casein genes (fig. 10).

Another conserved DNA fragment was present 25 bp upstream from the previous one (fig. 11).

It is tempting to imagine that these sequences of DNA are involved in the hormonal control of milk protein gene expression. To the best of our knowledge, this has not been demonstrated yet.

III. The cellular mechanism possibly involved in the transduction of the prolactin message to milk protein genes.

1) *Role of kinase C.* — Kinase C has been shown to be involved in the transmission of hormonal messages and phorbol esters strongly stimulate this enzyme like natural diacylglycerol (Nishizuka, 1986). In a previous work, we

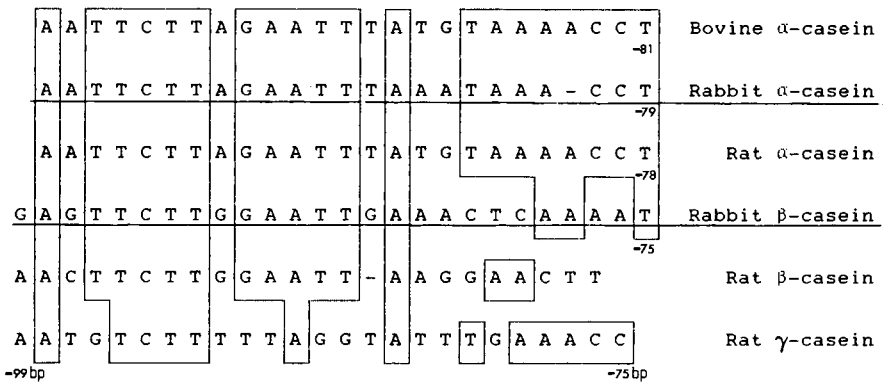

FIG. 11. — *Sequence homology between milk protein genes from various species in the - 100 to - 75 bp region.*

TABLE 1

Effect of phorbol esters and kinase C inhibitors on the induction of β-casein gene expression by prolactin.

Mammary explants were cultured for two days in the presence of insulin and cortisol (I + C). The agents and prolactin (PRL) were then added. After 8 h, tissue was collected and frozen. The presence of β-casein mRNA was evaluated using the corresponding oligolabelled DNA fragment as probe. Results which are means of triplicates are expressed as cpm of probe hybridized to filters loaded with 25 μg total RNA. (PdiBu : phorbol-12,13-dibutyrate, DPA : phorbol-12,13 diacetate, TPA : 12-O-tetradecanoylphorbol-13-acetate).

Hormones	I + C		I + C + PRL	
Agents	−	+	−	+
PdiBu (100 ng/ml)	141	—	980	126
PDA (100 ng/ml)	141	—	980	635
TPA (100 ng/ml)	141	—	980	1 042
H7 (50 μM)	141	—	980	1 415
W7 (50 μM)	141	—	980	1 439
PdiBu (100 ng/ml) + H7 (50 μM)	141	—	980	93
PdiBu (100 ng/mg) + W7 (50 μM)	141	—	980	89
Amiloride (250 μM)	100	266	697	702
Gossipol (100 μM)	100	—	697	842

observed that phorbol -12,13 dibutyrate (PdiBu) and, to a lower degree, 12-O-tetradecanoylphorbol -13-acetate (TPA) inhibited the differentiation of mammary cell by prolactin (Martel *et al.,* 1983a). Recent investigations have shown that in many tissues, kinase C is progressively down-regulated by phorbol esters or diacylglycerol. This problem was reassessed in the cultured mammary gland, with induction times not longer than 8 h.

Results of table 1 indicate that PdiBu was a strong inhibitor of prolactin action whereas at the same concentration (100 ng/ml) TPA, a strong tumor

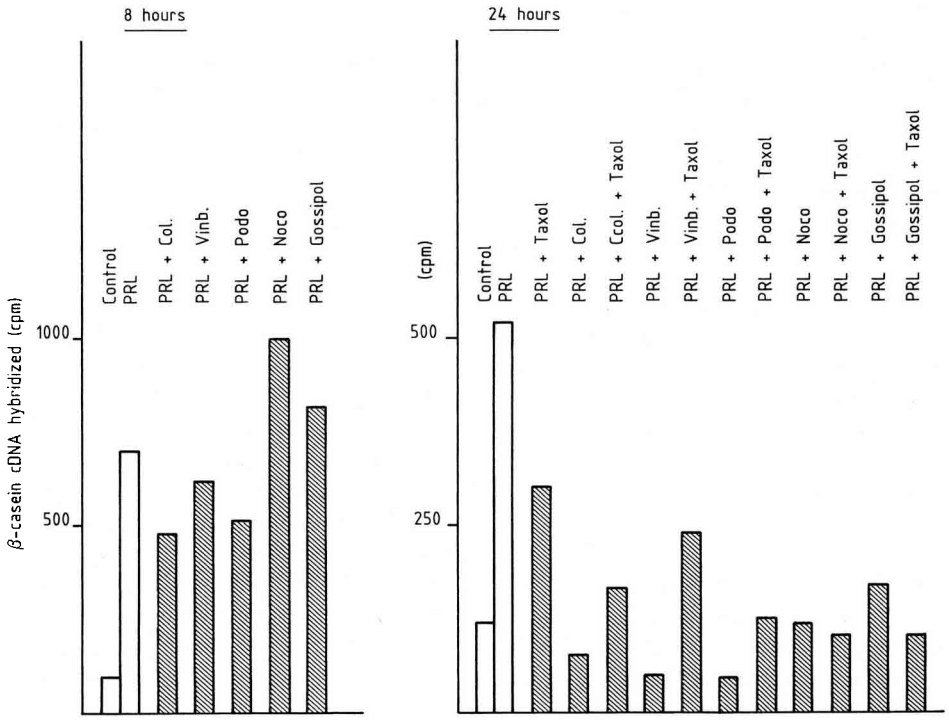

FIG. 12. — *Effect of various microtubule inhibitors and taxol on the induction of β-casein mRNA accumulation.* Mammary explants were cultured for two days in the presence of insulin (5 μg/ml) and cortisol (500 ng/ml). Prolactin (1 μg/ml) with or without drugs was then added and the culture was pursued 8 hours in one culture and 24 hours in the other. β-casein mRNA in the total RNA of the explants was then measured with a specific cDNA probe as depicted in fig. 1. Colchicine (col), vinblastine (vinb) and podophylotoxin (podo) were present at a concentration of 1 μM. The concentration of nocodazole (noco) and gossipol was 10 μM and that of taxol was 25 μM.

promoter, was inactive and PDA, an analogue devoid of promoter activity, was slightly inhibitory. The specific inhibitor of kinase C, H_7 (Hidaka *et al.*, 1984), did not inhibit prolactin action and did not abrogate the inhibitory effect of PdiBu. Data not shown here also indicate that similar conclusions can be drawn when induction is limited to 3 h. On the other hand ; parallel measurements of actin mRNA in the same samples revealed that no modification of this mRNA occurred under PdiBu and TPA action whereas PDA was slightly inhibitory (not shown). PdiBu therefore did not act through a general cytotoxic effect. Association of the calcium ionophore A23187 to phorbol esters did not change these conclusions (not shown). The compound W_7, which is a strong calmodulin inhibitor and a weak kinase C inhibitor (Hidaka *et al.*, 1984), did not prevent prolactin action. Similarly, amiloride which is an inhibitor of kinase C (Besterman *et al.*, 1985), did not prevent β-casein mRNA from accumulating under prolactin stimulation after 8 h (table 1). (In longer cultures, amiloride inhibits prolactin actions). In the same

way, gossipol, another kinase C inhibitor (Etindi and Rillema, 1987), inhibited prolactin action only after a long culture period (table 1 and figure 12). These data, indicating that there is no clear correlation between the activity of kinase C and the stimulation of casein gene expression, are in agreement with a recent report which shows that, in mouse mammary gland, kinase C is at its lowest level during lactation (Caufield and Bolander, 1986). The inhibitory effect of PdiBu is therefore most likely not due to genuine phorbol ester action. It might be due to the presence of the butyrate moiety which is a strong inhibitor of prolactin action (Martel *et al.*, 1983b).

2) *Role of phospholipid derivatives.* — Recent works carried out in various biological systems have pointed out the role of phospholipid derivatives and particularly of inositol triphosphate on the transduction of hormonal messages (Majerus *et al.*, 1986). Several inhibitors have been shown to block the cycle generating phosphatidylinositol. One of these inhibitors is neomycin which competes with phosphatidylinositol in its hydrolysis by phospholipase C (Carney *et al.*, 1985). Neomycin added with prolactin at a concentration of 1 mM inhibited strongly the hormonal action (table 2). Although neomycin acts on phospholipase C it is known to have other effects also related with phospholipid metabolism (Polascik *et al.*, 1987 ; Nakashima *et al.*, 1987). The experiment with neomycin thus does not strictly demonstrate that hydrolysis of phosphatidylinositol is required in prolactin action. Hexachlorocyclohexane has also been demonstrated to prevent phospholipase C from hydrolysing phosphatidylinositol (Ristow *et al.*, 1980 ; Hoffmann *et al.*, 1980). This compound was unable to alter prolactin action (table 2). LiCl prevents the recycling of inositol phosphate into phosphatidylinositol (Berridge, 1983). LiCl added with prolactin did not affect the induction of β-casein gene expression (table 2). The interpretation of the experiment with LiCl may be complicated by the fact that free inositol was present in medium 199 used in the cultures. In these conditions, recycling of inositol phosphate may be unnecessary for phosphatidylinositol phosphate to be regenerated (Ishihara *et al.*, 1987). Quercetin, which is an inhibitor of several kinases including phosphatidylinositol kinase (Sharoni *et al.*, 1986), kinase C (Gschwendt *et al.*, 1983) and a kinase found in mammary gland (Levy *et al.*, 1984) did not prevent prolactin from trigerring the accumulation of β-casein mRNA (table 2). Phosphatidic acid is a compound generated after hydrolysis of phospholipids by phospholipase C and it is a precursor of phospholipids. When added to culture medium, phosphatidic acid has been shown to mimic growth factor action for the accumulation of c-myc mRNA (Moolenaar *et al.*, 1986). When added with or without prolactin, phosphatidic acid did not mimic or alter hormonal response (table 2). Two other derivatives of phospholipid, phosphoethanolamine (table 2) and phosphocholine, were unable to mimic prolactin action (not shown). All these data argue rather in favour of the idea that phosphatidylinositides are not strictly involved in the mechanism of prolactin action. Obviously, additional experiments have to be performed before this possibility can be totally ruled out.

3) *Role of tyrosine kinase.* — Tyrosine kinase activity is associated with the receptor of several protein hormones and, in the case of insulin, it is involved in

TABLE 2

Effect of various agents related to phospholipid metabolism on the induction of β-casein gene expression by prolactin. Conditions of culture are those described in table 1. Results which are means of triplicates are expressed as cpm of β-casein cDNA probe hybridized to filters loaded with 25 μg total RNA.

Hormones	I + C		I + C + PRL	
Agents	−	+	−	+
Neomycine (1 mM)	126	—	772	218
LiCl (20 mM)	126	165	772	800
Hexachlorocyclohexane (30 μg/ml)	141	75	980	1 151
Quercetin (100 μM)	126	154	772	936
Phosphatidic acid (100 μM)	141	160	980	1 136
Phosphoethanolamine (10 mM)	100	140	980	—

TABLE 3

Effects of various agents interfering with phosphorylation on the induction of casein gene expression by prolactin. Conditions of culture are described in table 1. Results which are means of triplicates are expressed as cpm of β-casein probe hybridized to filters loaded with 25 μg total RNA.

Hormones	I + C		I + C + PRL	
Agents	−	+	−	+
Sodium orthovanadate (50 μM)	126	168	772	364
5'P-fluorosulfonyl benzoyl adenosine (100 μM)	126	140	772	631

the transmission of the hormonal message to glucose carrier (Ellis *et al.,* 1987). Tyrosine kinase activity is inhibited by 5'-p-fluorosulfonylbenzoyladenosine (Buhrow *et al.,* 1982), amiloride (Davis and Czech, 1985) and quercetin (Levy *et al.,* 1984). None of these compounds prevented prolactin action (table 2 and 3). On the other hand, sodium orthovanadate, which has been shown to mimic hormone action by specifically inhibiting tyrosine phosphatase (Klarlund, 1985 ; Green, 1986), did not mimic or stimulate prolactin action. On the contrary, it inhibited partly the accumulation of casein mRNA (table 3). These data support the view that a tyrosine phosphorylation is not necessary for prolactin to activate casein gene expression.

4) *Role of microtubules and tubulin.* — Previous works have shown that several tubulin binding drugs (colchicine, vinblastine, podophyllotoxin, nocodazole and tubulozole C) inhibit the induction of casein synthesis by prolactin (Servely *et al.,* 1987). One possible interpretation of these data was that microtubules are involved in the transmission of the prolactin message. More recently, it has been observed that estramustine, a microtubule-disrupting drug

which binds MAP but not tubulin, is unable to inhibit prolactin action (Zwierzchowski *et al.*, 1988). These experiments indicate that free tubulin, rather than tubulin organized in microtubules, is implicated in the prolactin mechanism of action. Results presented in figure 12 indicate that after 8 hours of action, the various microtubule-disrupting drugs, at most, only partially inhibited prolactin action, although in these conditions they rapidly disorganize the microtubular network (Ollivier-Bousquet, 1979). Taxol which is known to bind tubulin and to prevent disruption of microtobules by some of the drugs (Schiff and Horwitz, 1981) was slightly inhibitory for prolactin action and did not overcome the action of the drugs in all cases (fig. 12). The microtubule-disrupting drugs used here and taxol bind to specific sites on tubulin (Parness and Horwitz, 1981). The fact that taxol partially restores prolactin action in some cases might be due to some modifications of tubulin conformation independently of microtubule formation. These data provide additional support to the idea that microtubule integrity is not required for prolactin to act and that free tubulin rather than the microtubules themselves are involved in the transduction of the prolactin message.

Conclusion.

The data reported here indicate that three major rabbit milk protein genes, α_{s1}-casein, β-casein and WAP genes, are regulated by essentially similar hormonal control. The determination of the DNA structure in the upstream regions of the genes suggests that the conserved sequences are involved in the control of their transcription. The introduction, into cultured cells and into mouse and rabbit embryos, of hybrid genes containing these sequences has been undertaken tentatively to define the regulatory DNA sequences. These experiments and those aimed at determining the structure of the prolactin receptor should contribute to the understanding of the mechanism of prolactin action which still remains an enigma.

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Résumé. *Données récentes concernant le mécanisme de la régulation hormonale de l'expression des gènes des protéines du lait chez la lapine ; étude de la structure de ces gènes.*

Des explants et des cellules isolées de glande mammaire de lapin ont été cultivés en présence d'insuline, de prolactine et de cortisol seuls ou associés. Le contenu cellulaire en ARNm de la caséine-α_{s1}, de la caséine-β et de la whey acidic protein (WAP) a été évalué en utilisant les sondes ADNc correspondantes. Dans tous les cas, l'ARNm de la caséine-α_{s1}

est le plus abondant et celui de la WAP le moins abondant. Les trois gènes présentent pour l'essentiel une dépendance semblable vis-à-vis des hormones. La prolactine stimule l'accumulation des trois ARNm et l'insuline et le cortisol amplifient cette stimulation. Des fragments d'ADN génomique de lapin insérés dans le phage λ et contenant les séquences des gènes de la caséine-α_{s1}, de la caséine-β et de la WAP ont été clonés. La séquence primaire de ces gènes au voisinage du site CAP a été établie. Une comparaison des séquences situées en amont des sites CAP fait apparaître des analogies frappantes avec les gènes homologues de vache, de rat et de cobaye. Ceci suggère que ces séquences participent au contrôle transcriptionnel des gènes par les hormones. Le mécanisme impliqué dans la transduction du message prolactinique aux gènes des protéines du lait est inconnu. Avec l'aide des cultures d'explants mammaires, plusieurs mécanismes classiques de transduction ont été examinés. L'ester de phorbol, phorbol-12, -13-dibutyrate (PdiBu) inhibe l'action de la prolactine. Cependant, un autre promoteur de tumeur, le 12-O-tetradecanoyl phorbol-13-acétate (TPA) ne modifie pas l'action de la prolactine. L'inhibiteur H7 de la kinase C ne s'oppose pas à l'action de la prolactine et il ne lève pas l'inhibition du PdiBu. La kinase C n'est donc probablement pas essentielle pour que la transduction du message prolactinique aux gènes des protéines du lait ait lieu. La néomycine qui inhibe l'hydrolyse du phosphatidyl inositol par la phospholipase C empêche la prolactine d'agir, tandis que d'autres inhibiteurs restent sans effet. La dégradation du phosphatidyl inositol n'est probablement pas une étape essentielle dans l'action de la prolactine sur les gènes des protéines du lait. Des inhibiteurs de la tyrosine kinase et de la phosphatase ne modifient que modestement l'action de la prolactine. Les mécanismes de transduction utilisant l'activité tyrosine kinase ne sont donc probablement pas impliqués dans la transduction du message prolactinique à ses gènes cibles.

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