Expression of xanthine oxidoreductase in mouse mammary epithelium during pregnancy and lactation: regulation of gene expression by glucocorticoids and prolactin

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In the mammary gland of virgin mice, xanthine oxidoreductase (XOR) enzymic activity is barely measurable. A high increase in the levels of the enzyme is observed during the last days of pregnancy and during lactation, and this is parallelled by an elevation in the amounts of the respective protein and transcript. *In situ* hybridization experiments demonstrate that the XOR mRNA is specifically expressed in the alveolar epithelial cells of the mammary gland. In HC11 cells, a model culture system for normal breast epithelium, the levels of XOR enzymic activity are dose- and time-dependently induced by dexamethasone, and a further synergistic augmentation is observed in the presence of dexamethasone plus prolactin. Increased XOR gene expression is consequent on glucocorticoid receptor activation, as indicated by sensitivity to the specific receptor antagonist RU486. In addition, the phenomenon is likely to involve protein phosphorylation and dephosphorylation events, as suggested by modulation of XOR mRNA by tyrosine kinase and phosphatase inhibitors.

INTRODUCTION

Xanthine oxidoreductase (XOR) is the key enzyme in the catabolism of purines, catalysing the oxidation of hypoxanthine to xanthine and xanthine to uric acid. The protein is the product of a single gene [1] and it is localized in the cytoplasm of various cell types [2]. In its holo-enzymic form, XOR has a molecular mass of 300 kDa and consists of two identical 150 kDa subunits [3]. The enzyme is a molybdoflavoprotein, like sulphite oxidase and aldehyde oxidase [4–6], and it exists in two interconvertible forms, xanthine dehydrogenase (EC 1.1.1.204; XD) and xanthine oxidase (EC 1.1.3.22; XO) [7,8]. The former uses NAD^+ as the acceptor of reducing equivalents whereas the latter transfers them to molecular oxygen. XD can be converted into XO, either irreversibly by proteolysis, or reversibly through oxidation of crucial cysteine residues [9]. In various pathological conditions, the XO form of XOR is believed to play an important role in the processes that lead to tissue damage because of its ability to reduce oxygen to the toxic superoxide anion species [10–12]. On the other hand, under physiological conditions, XOR may have a cytoprotective action against local oxidative stress, since the final product of the activity of the enzyme is uric acid, a strong antioxidant [13].

Although in certain animal species the enzyme is particularly represented at the level of capillary endothelial cells [14,15], low but measurable amounts of XOR enzymic activity are found in various other cell types [2,16]. In the mouse, high levels of the protein are present in the enterocytes lining the proximal tract of the small intestine, as well as in a subpopulation of hepatocytes and in the alveolar cells of the lung [2]. Tissue- and cell-specific expression of the enzyme indicates that expression of the protein is highly regulated. Modulation of XOR gene expression by inflammatory mediators [16,17] further supports this contention. In fact, the XOR gene is transcriptionally upregulated by bacterial lipopolysaccharide, tumour necrosis factor and type I and type II interferon (IFN) in various mouse tissues [2] and mammalian cell lines [15,17].

Significant levels of XOR activity are found in the milk of humans and various other mammals. Indeed, human and cow milk is widely used as a rich source for purification of the enzyme [18–21]. XOR is produced in the mammary gland [14,22,23], however, the refined cellular localization, the endogenous stimuli and the molecular mechanisms responsible for the expression of XOR in mammary gland are not yet known. In this report, we first measured the levels of XOR enzymic activity, immunoreactive protein and mRNA in the mammary gland in virgin, pregnant and lactating mice at various stages during the gestation and lactation processes. Secondly, by *in situ* hybridization techniques, we determined the cells within the gland responsible for the synthesis of the XOR transcript. Thirdly, the expression of XOR was compared with that of a prototypical milk protein, i.e. β-casein. Finally, the hormones and the molecular mechanisms underlying XOR gene expression were studied in the *in vitro* model of lactogenesis represented by the normal mouse mammary epithelial cell line HC11.

MATERIALS AND METHODS

Animals and cell culture

To obtain pregnant and lactating mice, BDF_1 female mice weighing 18–20 g, obtained from Charles River Italia (Calco, Como, Italy), were mated in the central animal house facilities of the Istituto 'Mario Negri'. The day of pregnancy and lactation was calculated from the day of appearance of the vaginal plug. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance

* These two authors contributed equally to the generation of the data presented in this study.

Abbreviations used: XOR, xanthine oxidoreductase; XO, xanthine oxidase; XD, xanthine dehydrogenase; IFN, interferon; EGF, epidermal growth factor; G6PDH, glucose-6-phosphate dehydrogenase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

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Table 1 XOR activity, protein and mRNA levels in the mammary gland during mammogenesis and lactogenesis

XOR activity data are the means ± S.D. of three animals per experimental group. XOR protein levels are expressed relative to the value observed in virgin mice, which is taken as 1. Each experimental value is the mean ± S.D. of the densitometric analysis of Western blots obtained from four independent experiments. XOR mRNA data obtained from the densitometric analysis of Northern blots were normalized for the intensity of the 18 S rRNA in each sample. Results are expressed relative to the value observed in virgin mice, which is taken as 1. Each experimental value is the mean ± S.D. of four independent experiments. Statistical analysis was performed according to the Tukey's test following analysis of variance. * Significantly ($P < 0.01$) higher relative to the corresponding values observed in virgin mice, pregnant mice of 6, 12 and 18 days and post-lactating mice. † Significantly ($P < 0.01$) higher relative to the values observed in pregnant mice of 6 and 12 days and post-lactating mice. p.c., post-coitum.

Animals	XOR activity (m-units/mg)	XOR protein (relative amount)	XOR mRNA (relative amount)
Virgin mice	1.7 ± 0.1	1.0	1.0
Pregnant mice			
6 day p.c.	1.0 ± 0.6	1.4 ± 0.3	1.4 ± 0.9
12 day p.c.	1.6 ± 0.1	1.1 ± 0.5	3.6 ± 1.8
18 day p.c.	4.5 ± 1.0	6.6 ± 1.3†	58.8 ± 24.0†
Lactating mice			
1 day	11.5 ± 0.2*	8.0 ± 2.5†	56.3 ± 12.7†
2 days	10.0 ± 1.1*	6.9 ± 1.9†	55.7 ± 15.6†
3 days	11.1 ± 0.9*	7.8 ± 1.9†	50.3 ± 15.3†
7 days	14.8 ± 3.5*	11.3 ± 3.2†	31.5 ± 17.1†
Post-lactating mice	2.4 ± 0.5	1.7 ± 1.1	7.1 ± 1.2

with national (D. L. n. 116, G. U., suppl. 40, 18 Febbraio 1992) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, Dec. 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985).

HC11 cells, a gift from Dr. B. Groner (Institute for Experimental Cancer Research, Freiburg, Germany) and Dr. N. E. Hynes (Friedrich Miescher-Institut, Basel, Switzerland), were grown in RPMI 1640 (Gibco-BRL, Gaithersburg, MD, U.S.A.) supplemented with 10 % (v/v) fetal calf serum, 5 μg/ml insulin (Sigma, St. Louis, MO, U.S.A.) and 10 ng/ml epidermal growth factor (EGF; Sigma). For the induction of XOR, cells were plated at the density of 1.1×10^5 cells/cm^2, grown to confluency and left for a further 2 days in the absence of EGF. EGF-depleted cells were subsequently treated in the presence of 10^{-8} M dexamethasone alone or a combination of dexamethasone and ovine prolactin (5 μg/ml luteotropic hormone; Sigma), unless otherwise specified. Cultures were free from mycoplasma as assessed using the Hoechst 33258 fluorescent dye system (Farbwerke Hoechst AG, Frankfurt, Germany).

XOR enzymic assay

Tissues containing a pair of abdominal mammary glands (1 cm in diameter for a single mammary gland) were isolated from virgin mice, pregnant mice at days 6, 12 and 18 of gestation, lactating mice at days 1, 2, 3 and 7 following delivery, and mice that had been separated from pups for 10 days following a 3-week lactation period. Tissues were homogenized in 2–10 vol. of enzyme-extraction buffer containing 50 mM Tris/HCl (pH 8.0), 0.1 mM PMSF, 2 μM leupeptin and 0.15 μM aprotinin. HC11 homogenates were obtained following three cycles of freezing and thawing in the presence of the enzyme extraction buffer. Tissue and HC11 cell homogenates were centrifuged at 105000 ***g*** for 30 min or at 10000 ***g*** for 30 s respectively, and the corresponding supernatants were used for the XOR radiometric assay, which was performed as previously described [2]. One unit of XOR enzymic activity is defined as the amount of enzyme capable of transforming 1 μmol of hypoxanthine into xanthine and uric acid in 1 min at 37 °C. Proteins were determined according to the method of Bradford [24] using a commercially available kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

Western blot and Northern blot analysis

Tissue and cell homogenates prepared for the XOR assay were pooled, and proteins (100 μg) were electrophoresed on an 8 % SDS-denatured polyacrylamide gel. Conditions for blotting and detection of the specific XOR bands were as described previously [2]. Immunoreactive protein bands were revealed by a chemiluminescence-based procedure using an ECL detection kit (Amersham, Little Chalfont, U. K.) according to the instructions of the manufacturer.

Total RNA was prepared from mouse mammary glands, or from HC11 cells, according to standard procedures [25]. RNA (20 μg for the mammary gland tissue and 30 μg for HC11 cells) was fractionated on a 1 % agarose/6 % formaldehyde gel and blotted onto nylon membranes (GeneScreen Plus; New England Nuclear, Boston, MA, U.S.A.). Membranes were sequentially hybridized with the following ^{32}P-radiolabelled probes: a 2 kb fragment of the mouse XOR cDNA (Zap 64; [16]); a 525 bp mouse β-casein cDNA fragment (nucleotides 9278–9802; [26]), which was RT-PCR-amplified from HC11 RNA using synthetic oligonucleotides designed from published sequences [26]; and a synthetic oligonucleotide (5′-ACGGTATCTGATCGTCTTCG AACC-3′) that recognizes 18 S ribosomal RNA [27].

XOR mRNA signals were normalized to 18 S values obtained on the same blot to control for variation in loading and transfer among samples. The conditions for probe labelling and membrane hybridization were according to standard procedures [25]. Genistein and orthovanadate were purchased from Sigma, whereas Ru486 was a gift from Roussel UCLAF (Vitry-sur-Seine, France).

XOR protein and mRNA were quantified by densitometric analysis using an RAS 3000 videoimaging system (Amersham). Densitometry was performed at conditions of linearity for protein or mRNA content versus exposure time.

Nuclear transcription run-on assay

Nuclei were prepared from HC11 cells that were harvested 30 h after the appropriate treatment. The nuclear run-on reaction was performed according to the method described previously [17]. The cDNAs used for this experiment were those coding for mouse liver XOR cDNA (zap64; [16]), glucose-6-phosphate dehydrogenase (G6PDH; [28]), histone H2a [29] and glyceraldehyde-3-phosphate dehydrogenase (G3PDH; [30]).

In situ hybridization

The 2 kb mouse XOR cDNA fragment, Zap 64 [16], and the 525 bp PCR-amplified mouse β-casein cDNA were subcloned in the plasmid vector pBluescript (Stratagene, La Jolla, CA, U.S.A.), and used as templates for the synthesis of sense and antisense riboprobes, employing T3 and T7 RNA polymerases (Stratagene), in the presence of [^{35}S]thio-UTP (specific radioactivity 1200 Ci/mmol; Amersham). Template DNAs were degraded by DNase I (Pharmacia, Uppsala, Sweden), and the average length of the riboprobes was adjusted to approx. 150 nucleotides by alkaline treatment [31]. The mouse tissues containing abdominal mammary glands and muscle were cut in

circles (1 cm in diameter), fixed in 4% paraformaldehyde overnight, embedded in paraffin, sectioned to 5 μm thickness and mounted on chromalum-containing gelatin-coated slides.

HC11 cells were plated on tissue-culture chambers (Lab-Tek Chamber Slides, Nunc Inc., Naperville, IL, U.S.A.) at a density of 1.1×10^5 cells/cm^2 and grown to confluency for 24 h. Following depletion of EGF from the culture medium for 2 days, treatments with the appropriate stimuli were performed. At the end of each treatment, cells were fixed in 4% paraformaldehyde at 4 °C for 3 h.

The conditions for the pretreatment of slides, hybridization, washing and detection by the nuclear track emulsion technique have been described precisely in a previous report [2]. At the end of the *in situ* hybridization, the mammary gland tissue sections were stained with haematoxylin–eosin, whereas HC11 cells were stained with Giemsa.

RESULTS

Modulation of XOR activity and protein levels in mammary gland during pregnancy and lactation

XOR enzymic activity was measured in the mammary gland of virgin, pregnant and lactating mice. As shown in Table 1 virgin mice have very low but detectable amounts of XOR activity. A slight, albeit statistically insignificant, increase in enzymic activity is observed at late gestation, and the levels of XOR activity are further and significantly augmented (6–8-fold relative to the amounts observed in virgin mice) during lactation. Following a 3-week feeding period, if mothers are separated from pups, within 10 days, the level of XOR activity goes back to that observed in virgin animals. In the mammary gland, the proportion of XOR present in the XO form is always constant (50–60% of XD + XO) irrespective of the experimental conditions considered (results not shown). No significant variations in the absolute amounts of XOR enzymic activity were observed in liver during mammogenesis or lactogenesis relative to that

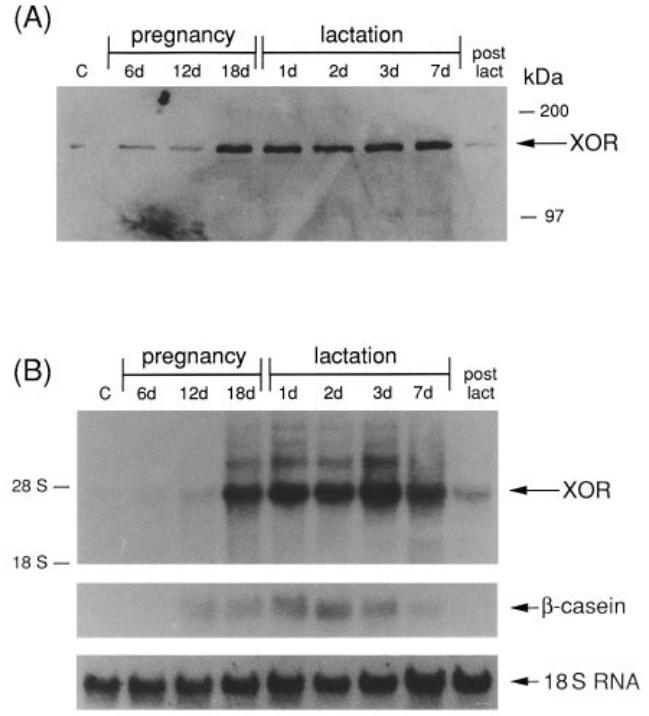

Figure 1 Western and Northern blot analysis of XOR protein and mRNA in mouse mammary glands during pregnancy and lactation

Mammary glands were isolated from virgin mice (C), pregnant mice at 6, 12 and 18 days of gestation, lactating mice at 1, 2, 3 and 7 days after delivery, and post-lactating mice (post lact). (**A**) Protein extracts (100 μg/lane) were electrophoresed and subjected to Western blot analysis. The XOR immunoreactive band (150 kDa) is indicated by an arrow and the position of the molecular-mass markers is indicated on the right (myosin, 200 kDa; phosphorylase *b*, 97 kDa). A representative blot of four independent experiments is shown. (**B**) Total RNA (20 μg/lane) was electrophoresed and subjected to Northern blot analysis. The XOR mRNA is indicated by an arrow on the right and the positions of 18 S and 28 S ribosomal RNAs are shown on the left. The same blot was sequentially hybridized with a mouse XOR cDNA, a β-casein cDNA fragment and an oligonucleotide-recognizing 18 S ribosomal RNA. A representative blot of four independent experiments is shown.

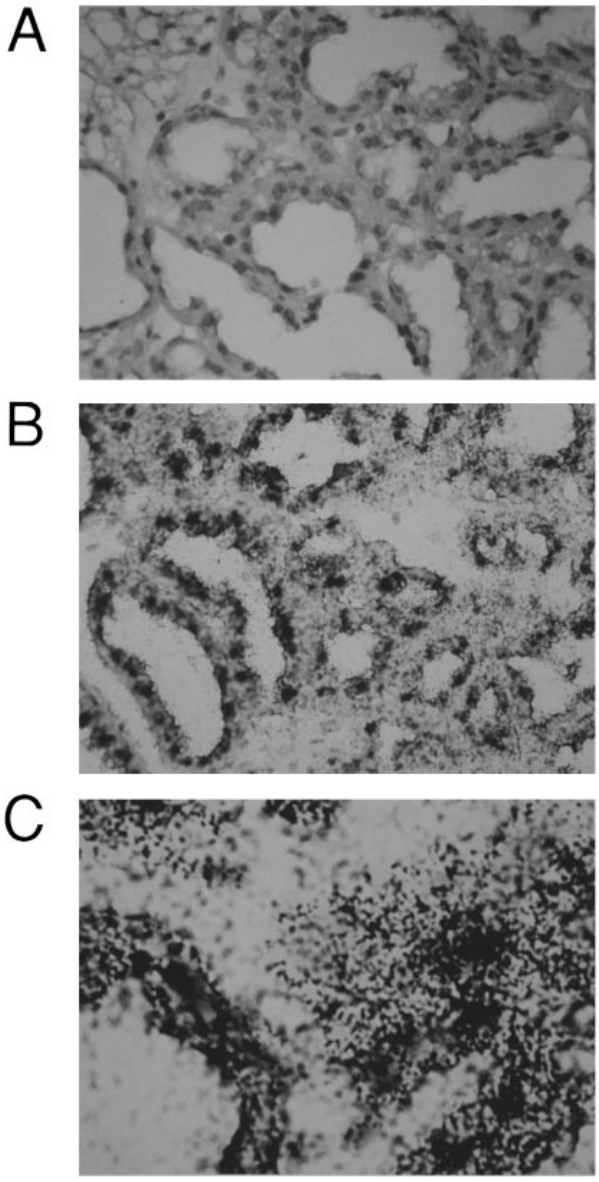

Figure 2 *In situ* hybridization of XOR mRNA in mammary glands during lactation

Mammary gland tissue was obtained from lactating mice 2 days (**A** and **B**) or 7 days (**C**) after delivery. Tissue sections were hybridized either with sense (**A**) or antisense (**B** and **C**) XOR cRNA. The magnification of (**A** and **B**) is $1.25 \times 6.3 \times 40$, whereas that of (**C**) is $1.25 \times 10 \times 100$.

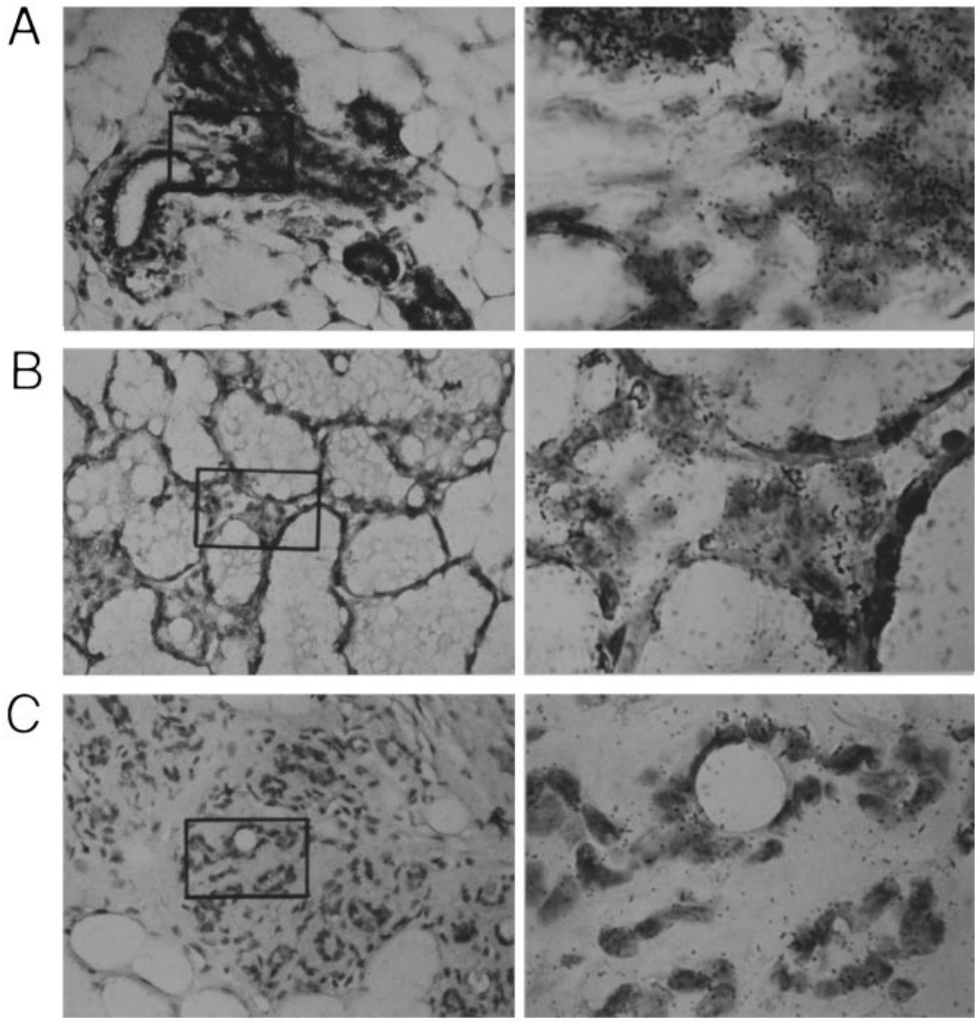

Figure 3 ***In situ*** **hybridization of XOR mRNA in mammary glands at different stages of mammogenesis and lactogenesis**

Mammary gland tissue was obtained from pregnant mice at 12 days (**A**) and 18 days of gestation (**B**), and from mice that had been separated from pups for 10 days following a 3-week lactation period (**C**). All the sections were probed with antisense XOR cRNA. The magnification of the photographs shown on the left side of each panel is $1.25 \times 6.3 \times 40$, whereas that on the right side is $1.25 \times 10 \times 100$. The field shown on each photograph at higher magnification (right) is boxed on the respective photographs at lower magnification (left).

observed in virgin (4.2 ± 1.8 m-units/mg protein; mean $\pm$ S.D., $n = 3$) and post-lactating (5.4 ± 0.3 m-units/mg protein; mean $\pm$ S.D., $n = 3$) animals.

To investigate whether the increase in XOR activity observed during lactation is concordant with an augmentation in the levels of the correponding protein, we performed Western blot experiments. A representative blot is shown in Figure 1(A), whereas quantitative results from four independent experiments are presented in Table 1. In virgin mice, a single band of approx. 150 kDa, corresponding to the XOR monomeric subunit, is recognized by a specific polyclonal antibody raised against the purified enzyme [2]. In pregnant mice, the amounts of XOR immunoreactive protein are significantly increased at day 18 of gestation, and these high levels of XOR protein are maintained throughout lactation. Ten days after the end of the feeding period, the amounts of the molybdoflavoprotein tend to decrease to those observed in virgin animals. Thus, during the development of the mammary gland and the secretory phase of lactation, quantitative changes in the levels of XOR activity are parallelled by similar quantitative alterations in the amounts of the relevant immunoreactive protein. However, assembly of the holoenzyme from the apoprotein may require some time, as suggested by significant accumulation of the XOR immunoreactive protein by day 18 of gestation, a time at which XOR enzymic activity starts to increase but is not yet significantly higher than that observed in virgin mice. This is not an unusual effect *in vivo*, and it was observed in certain mouse tissues upon induction of XOR by other stimuli like lipopolysaccharide and poly(I/C) [2,16]. Due to the complexity of the XOR holoenzyme, it is possible that one or more cofactors are present in the developing mammary gland at concentrations that limit the conversion of the apo-protein into the corresponding holoenzyme.

XOR gene expression in mammary glands during pregnancy and lactation

To study the molecular mechanisms underlying the up-regulation of XOR activity and protein levels, Northern blot analysis was performed on RNA isolated from mammary glands of virgin, pregnant and lactating mice. A representative blot is shown in

Figure 1(B) and quantitative results obtained from four independent experiments are summarized in Table 1. In virgin mice, very low levels of a single 4500 nucleotide-long XOR transcript are observed. A dramatic increase in the accumulation of this mRNA is observed in the mammary gland at day 18 of gestation. The levels of the transcript are high until day 7 of the lactation period, when expression of the mRNA tends to decrease. Following post-lactation involution of the mammary gland, the levels of the XOR transcript are significantly down-modulated. As shown on the middle panel of Figure 1(B), the transcript coding for one of the most abundant milk proteins, β-casein, is not expressed in the mammary gland of virgin mice. A specific β-casein mRNA band appears at day 12 of pregnancy, and the levels of this transcript increase at day 18 and remain constant throughout lactation. The transcript disappears in the post-lactation period. Thus, regulation of the XOR mRNA during mammogenesis and lactogenisis is consistent with that of the corresponding protein, and similar to the regulation of the β-casein gene.

To define cell type(s) responsible for the expression of the XOR gene, *in situ* hybridization experiments were performed on mammary gland sections obtained at different stages during the processes of mammogenesis and lactogenesis, using appropriate sense and antisense riboprobes synthesized from the XOR cDNA. As shown in Figure 2, the silver grains detected on the tissue sections are the result of specific hybridization of the anti-sense riboprobe to the XOR mRNA. In fact, no grain accumulation is observed on vicinal sections hybridized with an equally labelled sense-strand riboprobe (compare panels A and B). As expected from the results obtained from Northern blot experiments, XOR mRNA is almost undetectable on sections derived from virgin mice (results not shown) and from mice that discontinued breast feeding (Figure 3C). The XOR gene starts to be expressed in pregnant mice at day 6 of gestation, as demonstrated by the small but significant number of silver grains found on the epithelium of the growing and differentiating mammary gland (results not shown). During pregnancy, at days 12 and 18, substantial amounts of the XOR message are evident only in the epithelial cells which will form the internal part of the alveoli following completion of the cellular differentiation process (Figures 3A and 3B). The number of silver grains associated with the alveolar epithelial component of the mammary gland is increased dramatically during lactation, as shown in Figure 2(B) (2 days after delivery) and Figure 2(C) (7 days after delivery).

Similar experiments, using β-casein riboprobes, were performed on mammary gland sections adjacent to those used for XOR in order to verify the possible co-localization of the transcript coding for this prototypic milk protein and XOR mRNA. Whereas no β-casein mRNA expression is observed in virgin and post-lactating mice, during the late phases of pregnancy and during lactation, specific silver grains are strictly localized on the epithelial cells forming the internal part of the secretory alveoli (results not shown). Thus our data demonstrate that the two transcripts are synthesized by the same type of cells.

Hormonal regulation of XOR enzymic activity and protein and mRNA levels in HC11 cells

To investigate the stimuli responsible for the induction of XOR in the mammary gland and to evaluate their mode of action, a mouse cell line, HC11, derived from mammary gland epithelial cells [32], was chosen for further experiments. The growth of HC11 cells is EGF-dependent, however, lactogenesis (as assessed by the measuremenet of β- and γ-caseins) is observed only in confluent cultures depleted of the growth factor and treated with a mixture of dexamethasone and prolactin [32,33].

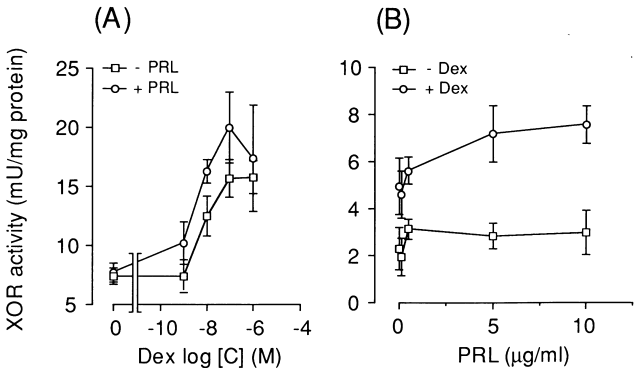

Figure 4 Prolactin (PRL) and dexamethasone (Dex) dose-dependent induction of XOR activity in HC11 cells

Confluent and EGF-depleted HC11 cells (1×10^6cells/2 ml) were incubated for 2 days in medium containing the indicated amounts of Dex in the absence (−PRL) or in the presence (+PRL) of PRL at a concentration of 5 μg/ml (**A**), or in medium containing the indicated amounts of PRL in the absence (−Dex) or in the presence (+Dex) of Dex at a concentration of 10^{-8} M (**B**). At the end of each treatment, cells were were processed for XOR assays. The results are the means ± S.D. of three separate cultures.

During the logarithmic phase of growth of HC11 cells, no significant XOR activity is measurable (results not shown), whereas confluent and contact-inhibited cells express detectable amounts of the enzymic activity. As shown in Figure 4(A), XOR activity is inducible following treatment with dexamethasone for 2 days. The effect of dexamethasone is dose-dependent at concentrations of the corticosteroid between 10^{-6} and 10^{-8} M. Whereas treatment with prolactin (5 μg/ml) alone is ineffective in inducing XOR enzymic activity, contemporaneous treatment of HC11 cells with the lactogenic glycoprotein in combination with dexamethasone leads to enhanced expression of the enzyme relative to treatment with the corticosteroid alone. Maximal synergism between prolactin and dexamethasone is observed when the steroid is present at a concentration of 10^{-8} M. As shown in Figure 4(B), at this concentration of dexamethasone, prolactin enhances XOR induction in a dose-dependent manner which tends to plateau at around 5 μg/ml. This is the concentration of prolactin which is generally used to obtain optimal induction of milk protein synthesis in HC11 cells [32]. Thus, unless otherwise stated, all the subsequent experiments were performed in medium containing 10^{-8} M dexamethasone and 5 μg/ml prolactin. The following points are worth mentioning. First, XOR induction by dexamethasone is not influenced by the presence of EGF in the medium, whereas synergistic up-regulation of the molybdoflavoprotein by the combination of dexamethasone and prolactin is inhibited by the growth factor (results not shown). Secondly, the proportion of XOR present in the XO form is between 35 and 40 % under basal conditions and it is not altered upon treatment with dexamethasone, prolactin or the combination of the two compounds at any time point. Thirdly, induction of XOR is observed, not only in the presence of the synthetic corticosteroid dexamethasone, but also in the presence of cortisol, a natural glucocorticoid. In fact, treatment of HC11 with 10^{-8} and 10^{-6} M cortisol for 3 days increases the basal level of XOR activity 2.0- and 3.5-fold respectively. Moreover, addition of prolactin (5 μg/ml) to medium containing 10^{-6} M cortisol results in a 5-fold induction of XOR relative to control conditions.

Table 2 Time-dependent induction of XOR activity, protein and mRNA by dexamethasone (Dex) and Dex + prolactin (PRL) in HC11 cells

Confluent and EGF-depleted HC11 cells were incubated in medium alone, or in medium containing Dex (10^{-8} M), PRL (5 μg/ml) or a combinatin of Dex and PRL for the indicated amount of time. XOR enzymic activity data are the means ± S.D. of three separate culture dishes. XOR protein levels are expressed relative to the value observed in control cultures at time zero, which is taken as 1. Each experimental value is the mean ± S.D. of the densitometric analysis of Western blots obtained from four independent experiments. XOR mRNA data obtained from the densitometric analysis of Northern blots are normalized for the intensity of the 18 S rRNA in each sample. Results are expressed relative to the value observed in control cultures at time zero, which is taken as 1. Each experimental value is the mean ± S.D. of four independent experiments. Statistical analysis was performed according to the Tukey's test following analysis of variance. * Significantly higher ($P < 0.01$) relative to all the time points of control cultures. † Significantly higher ($P < 0.01$) relative to medium containing PRL at same time point. ‡ Significantly higher ($P < 0.01$) relative to the medium containing Dex at same time point. § Significantly higher ($P < 0.05$) relative to the medium containing PRL at the same time point. *‖ Significanatly higher ($P < 0.05$) relative to the medium containing Dex at the same time point. N.D., not determined.

Treatment time (h)	XOR activity (m-units/mg protein)	XOR protein (relative amount)	XOR mRNA (relative amount)
Medium			
0	2.0 ± 0.4	1.0	1.0
8	N.D.	N.D.	0.8 ± 0.2
24	2.1 ± 0.3	0.9 ± 0.3	0.7 ± 0.4
48	N.D.	N.D.	1.0 ± 0.2
72	1.8 ± 0.3	0.7 ± 0.1	1.3 ± 0.2
120	3.0 ± 0.7	1.6 ± 0.6	N.D.
PRL			
8	N.D.	N.D.	0.8 ± 0.1
24	2.1 ± 0.3	0.6 ± 0.1	0.7 ± 0.2
48	N.D.	N.D.	1.1 ± 0.2
72	3.2 ± 1.2	0.8 ± 0.2	2.4 ± 0.3
120	3.9 ± 0.7	1.6 ± 0.8	N.D.
Dex			
8	N.D.	N.D.	1.2 ± 0.3
24	2.8 ± 0.2	2.0 ± 0.8	2.4 ± 0.7
48	N.D.	N.D.	4.0 ± 0.8*†
72	3.5 ± 0.7	2.2 ± 1.6	4.9 ± 1.2*§
120	8.6 ± 0.5*†	4.0 ± 1.8	N.D.
Dex + PRL			
8	N.D.	N.D.	1.5 ± 0.5
24	3.4 ± 0.8	2.1 ± 0.9	2.9 ± 1.0
48	N.D.	N.D.	6.6 ± 1.5*†‖
72	5.2 ± 0.7*†‖	4.5 ± 3.4	7.5 ± 3.2*†‖
120	15.7 ± 0.9*†‡	5.2 ± 1.2*†‖	N.D.

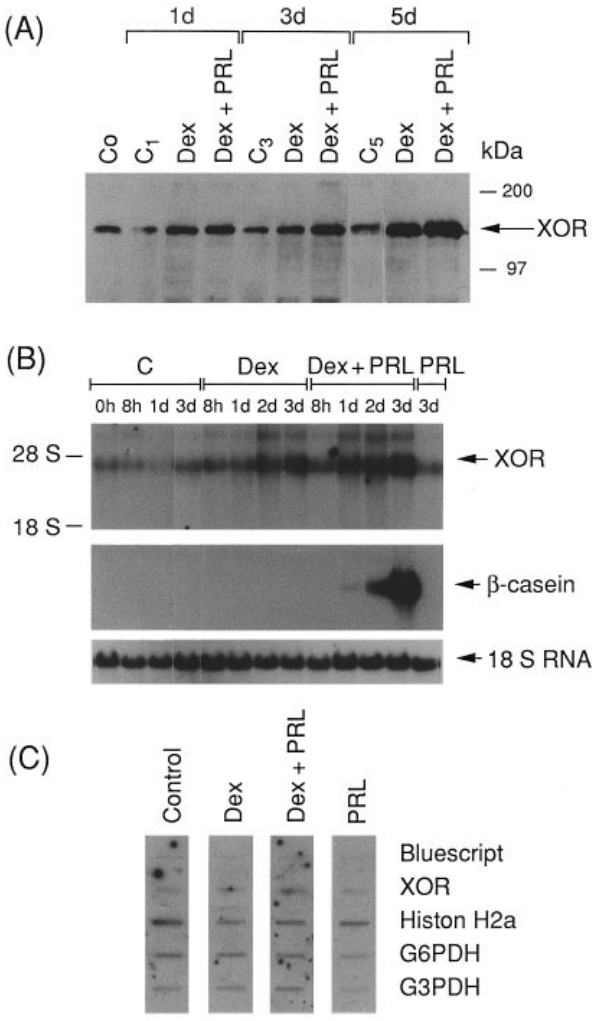

Figure 5 Time-dependent up-regulation of XOR activity, protein, mRNA and gene transcription by dexamethasone (Dex) and prolactin (PRL) in HC11 cells

Confluent and EGF-depleted HC11 cells were incubated in medium alone (C), or in medium containing Dex (10^{-8} M), PRL (5 μg/ml) or the combination of Dex and PRL for the indicated amount of time in days (d). (**A**) Protein extracts (100 μg/lane), derived from three dishes for each experimental group, were subjected to Western blot analysis. The 150 kDa immunoreactive XOR protein band is indicated by an arrow and the positions of molecular-mass markers are indicated on the right (myosin, 200 kDa; phosphorylase *b*, 97 kDa). A representative blot of four independent experiments is shown. (**B**) RNA (30 μg/lane) was electrophoresed and subjected to Northern blot analysis. The same blot was sequentially hybridized to mouse XOR cDNA, β-casein cDNA and an oligonucleotide recognizing 18 S ribosomal RNA. The positions of 18 S and 28 S ribosomal RNAs are shown on the left. A representative blot of four independent experiments is shown. (**C**) Run-on experiments were performed on nuclear preparations incubated with [^{32}P]UTP. Radiolabelled transcripts were hybridized to the following probes: Bluescript, mouse XOR cDNA, histone H2a cDNA, G6PDH cDNA and G3PDH cDNA.

Table 2 illustrates that the induction of XOR activity by dexamethasone alone, or by the combination of dexamethasone and prolactin, is a relatively slow process. Upon treatment of HC11 cells with dexamethasone or the combination of dexamethasone and prolactin, the levels of XOR activity increase in a time-dependent manner and they reach a maximum at day 5. Treatment with dexamethasone or the combination of the steroid and prolactin augments XOR activity approx. 4- and 8-fold respectively, over the basal level of the enzyme observed at time zero. A similar time-dependent augmentation of XOR activity is evident when HC11 cells are treated with dexamethasone alone at a concentration of 10^{-6} M, however, under these conditions, strong stimulation by the corticosteroid alone tends to mask the synergism with prolactin (results not shown). Treatment of cells with prolactin alone does not significantly affect the basal level of XOR expression at any time point. Notice that, at least until day 5 of culture in the absence of EGF, HC11 cultures contain more than 90% of viable cells, and this level of cell viability is not altered by treatment with dexamethasone, prolactin or the combination of the two compounds. Thus increases in XOR following treatment with dexamethasone or dexamethasone plus prolactin are due to a *bona fide* induction process.

As demonstrated by the representative Western blot analysis shown in Figure 5(A) and the quantitative data presented in Table 2, the increases in XOR activity triggered by dexamethasone alone or the combination of dexamethasone and prolactin are accompanied by a quantitatively similar augmentation in the levels of the corresponding immunoreactive protein. The time course for the increase in the XOR immunoreactive protein

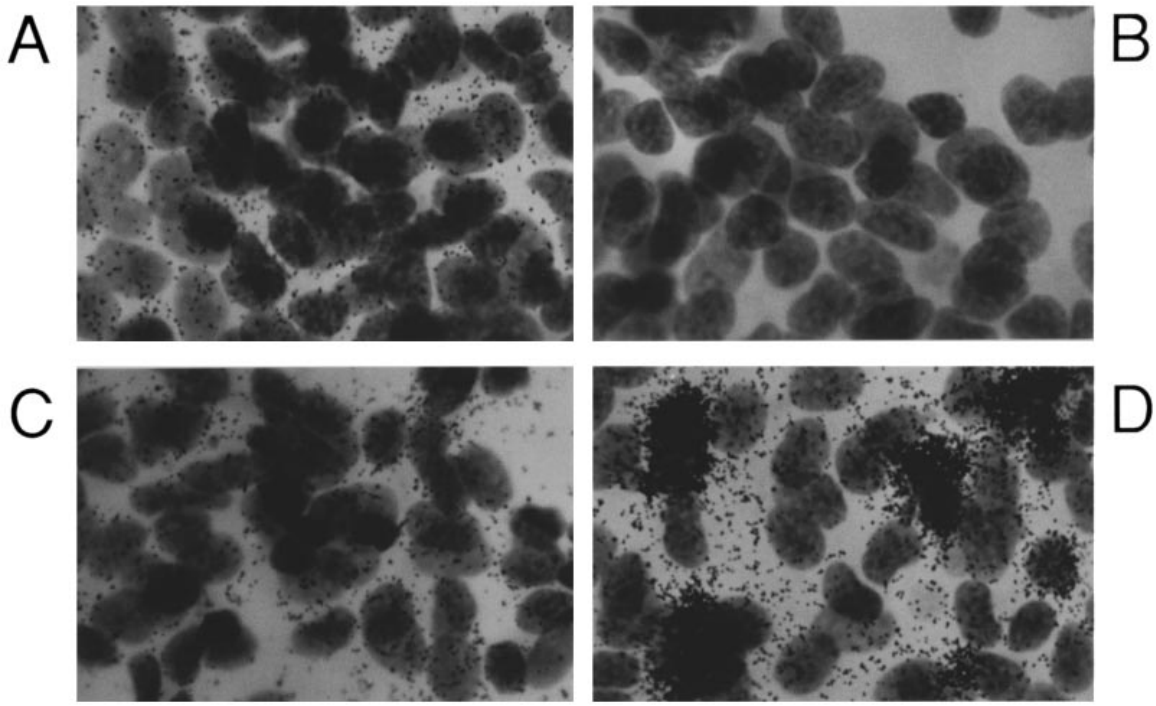

Figure 6 ***In situ*** **hybridization of XOR and β-casein mRNAs in HC11 cells treated with dexamethasone (Dex) and prolactin (PRL)**

Confluent and EGF-depleted HC11 cells grown on chamber slides were treated in medium containing Dex (**A** and **B**) or the combination of Dex and PRL (**C** and **D**) for 3 days. Following each treatment, cells were fixed and processed for *in situ* hybridization. The ribo-probes used were antisense XOR cRNA (**A** and **C**) and antisense β-casein cRNA (**B** and **D**). The magnification of the photographs is $1.25 \times 10 \times 100$.

level is similar to that of the rcorresponding enzymic activity. This indicates that, in HC11 cells, assembly of the holoenzyme from the apoprotein is almost immediate.

As shown in Figure 5(B) and Table 2, at confluency HC11 cells contain detectable amounts of XOR mRNA of a size identical with that observed in the epithelial cells of the mammmary gland *in vivo*. Upon treatment with dexamethasone alone, the levels of the XOR transcript increase and a further enhancement is observed in the presence of the combination of the corticosteroid and prolactin. In both experimental situations, a significant up-regulation of the mRNA is observed between days 2 and 3 from the beginning of the treatment. This demonstrates that increased accumulation of the XOR transcript precedes induction of the enzyme. The kinetics of XOR mRNA accumulation are similar to those of the β-casein transcript. In fact, the latter message becomes evident when the HC11 cells are kept in contact with both dexamethasone and prolactin for 2 days.

Accumulation of XOR mRNA is mainly a consequence of the transcriptional activation of the corresponding gene, as demonstrated by the nuclear run-on experiment represented in Figure 5(C). In HC11 cells, the basal level of XOR gene transcription is very low and just above background. Prolactin treatment does not affect the transcriptional activity of the gene. Upon challenge of HC11 cells with dexamethasone, an increase in the rate of XOR gene transcription is observed. This increase is further enhanced by addition of prolactin to the corticosteroid-containing cell growth medium. The effects of prolactin, dexamethasone or the combination of dexamethasone plus prolactin on the rate of transcription of the histone H2a, G6PDH and G3PDH genes are minimal, and support the specificity of the phenomena observed at the level of the XOR gene.

To examine whether the time-dependent increase in the XOR transcript is simply due to a general response of all the cells to dexamethasone and prolactin, or to the response of a particular subpopulation, *in situ* hybridization was performed on HC11 cultures after appropriate treatments for 1 and 3 days. The results obtained from 3-day treatments are illustrated in Figure 6. Under basal conditions, the whole population of HC11 cells express low but detectable amounts of the XOR transcript. Upon induction by dexamethasone alone, or by the combination of dexamethasone and prolactin, the number of silver grains increases in a time-dependent manner (Figures 6A and 6C), as expected from the results of the Northern blot analysis. The silver grains are uniformely distributed on all the cells present in the culture dishes. This is different from what is observed for the β-casein transcript. The levels of the milk protein mRNA are augmented upon induction with the combination of dexamethasone and prolactin (but not with dexamethasone alone) (Figures 6B and 6D) in a time-dependent manner, however, the silver grains accumulate only in a small fraction of the cultured cells, which probably represents the differentiated subpopulation responsible for the production of milk (Figure 6D).

Effects of cycloheximide, actinomycin D, orthovanadate, genistein and RU-486 on XOR mRNA in HC11 cells

To investigate in more detail the molecular mechanisms underlying the regulation of XOR gene transcription by dexamethasone and the combination of dexamethasone plus prolactin, Northern blot experiments were performed on RNA prepared from HC11 cells treated with a series of compounds affecting cell homoeostasis. A representative RNA blot of four independent experi-

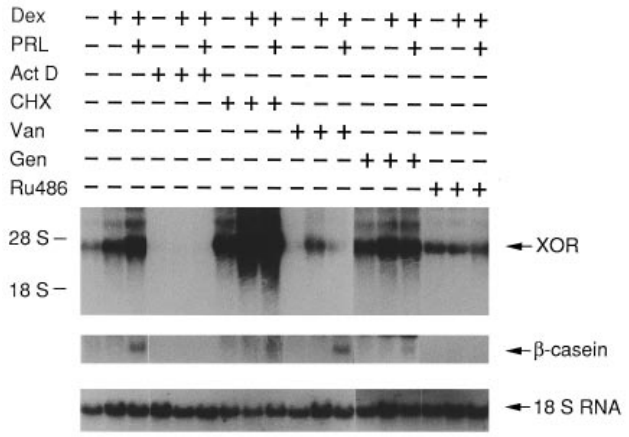

Figure 7 Effects of actinomycin D (Act D), cycloheximide (CHX), orthovanadate (Van), genistein (Gen), and RU486 on the induction of XOR mRNA by dexamethasone (Dex) and prolactin (PRL)

Confluent and EGF-depleted HC11 cells were treated with Dex or the combination of Dex and PRL for 30 h in the presence of Act D (2 μg/ml), CHX (10 μg/ml), Van (20 μM), Gen (10 μM) or RU486 (0.1 μM). At the end of each treatment, RNA (30 μg/lane) extracted from the cells was subjected to Northern blot analysis. The same blot was sequentially hybridized with XOR and β-casein cDNA fragments and the oligonucleotide that recognizes 18 S ribosomal RNA. The position of each transcript is indicated by an arrow on the right, and that of 28 S and 18 S ribosomal RNA is shown on the left. A representative blot of four independent experiments is shown.

ments is shown in Figure 7 and the quantitative results are summarized in Table 3. Actinomycin D, an RNA polymerase II inhibitor, completely inhibits the up-regulation of XOR mRNA observed in the presence of dexamethasone or the combination of dexamethasone plus prolactin. In addition, the (dexamethasone plus prolactin)-dependent up-regulation of the β-casein mRNA is completely blocked by actinomycin D. Although these effects are evident at a concentration of actinomycin D that does not affect the viability of cells, decreases in the levels of the transcripts coding for constitutively expressed genes such as G6PDH and alpha-actin are also observed (results not shown). Thus, in HC11 cells, the inhibitory effects of actinomycin D on inducible and RNA polymerase II-dependent genes may not be completely specific. Nevertheless, the data are in line with the results obtained by nuclear run-on experiments, and they suggest that increases in the levels of expression of the XOR gene are mainly the result of transcriptional events. Complete inhibition of protein synthesis by cycloheximide, at a concentration that does not cause a decrease in cell viability, results in increased accumulation of the XOR transcript over that observed in HC11 cells incubated in medium alone. When the protein synthesis inhibitor is added to the medium containing dexamethasone or dexamethasone plus prolactin, a similar enhancing effect on the induction of the XOR transcript is observed. This suggests the presence of short-lived protein(s), which control XOR gene expression in a negative fashion. Inhibition of protein synthesis blocks the up-regulation of β-casein mRNA afforded by treatment of HC11 cells with dexamethasone plus prolactin. Figure 7 and Table 3 also document that orthovanadate, a general inhibitor of intracellular phosphatases, disrupts the synergism between the corticosteroid and prolactin. The same compound does not affect β-casein gene expression. By contrast, an enhancement in the dexamethasone-induced expression of XOR is observed when HC11 cells are treated with the relatively specific tyrosine kinase inhibitor, genistein. Treatment of HC11 cells

Table 3 Effects of actinomycin D (ActD), cycloheximide (CHX), orthovanadate (Van), genistein (Gen) and Ru486 on XOR mRNA induction by dexamethasone (Dex) and Dex + prolactin (PRL) in HC11 cells

Confluent and EGF-depleted HC11 cells were cultured for 30 h in the presence of the indicated compounds. XOR mRNA quantification is as described in the legend to Table 2. Data are expressed relative to the amount of XOR mRNA observed in HC11 cells cultured in medium alone (taken as 1), and presented as mean relative amounts $\pm$ S.D. of four independent experiments. Statistical analysis was performed according to Tukey's test following analysis of variance. * Significantly higher ($P < 0.01$) relative to Dex. † Significantly higher ($P < 0.05$) relative to Dex. ‡ Significantly higher ($P < 0.01$) relative to Dex + PRL. § Significantly lower ($P < 0.01$) relative to Dex + PRL.‖ Significanatly lower ($P < 0.05$) relative to Dex.

Treatment	XOR mRNA (relative amounts)
Medium	1.0
Dex	3.4 ± 1.5
Dex + PRL	7.9 ± 2.0*
ActD	< 0.1
ActD + Dex	< 0.1
ActD + Dex + PRL	< 0.1
CHX	24.5 ± 13.1
CHX + Dex	36.2 ± 22.1†
CHX + Dex + PRL	53.4 ± 34.1‡
Van	1.4 ± 0.9
Van + Dex	2.2 ± 0.3
Van + Dex + PRL	1.4 ± 1.1§
Gen	1.1 ± 0.5
Gen + Dex	7.1 ± 1.7*
Gen + Dex + PRL	6.5 ± 2.7
Ru486	1.3 ± 0.6
Ru486 + Dex	1.5 ± 0.8‖
Ru486 + Dex + PRL	2.0 ± 0.4§

with genistein does not have a significant effect on the level of expression of the XOR gene under basal conditions. The compound increases the accumulation of the XOR transcript observed in the presence of the corticosteroid 2-fold, however, it inhibits the enhancement of XOR mRNA accumulation caused by prolactin, when the lactogenic hormone is combined with dexamethasone. Similar results were obtained when genistein was substituted by erbstatin, another tyrosine kinase inhibitor (results not shown). Under the same conditions, genistein reduces the up-regulation of β-casein gene expression caused by the combination of dexamethasone and prolactin. Finally, the steroid receptor antagonist, Ru486, almost completely blocks the induction of XOR and β-casein transcripts caused by dexamethasone or dexamethasone plus prolactin.

DISCUSSION

In this report, we demonstrate that XOR, the key enzyme in the catabolism of purines, is induced and expressed at high levels in the mouse mammary epithelium during the late stages of pregnancy and throughout the lactation period. XOR is a very complex enzyme and its intracellular accumulation is potentially regulated at various levels [2,34]. In the mammary gland, induction of the enzyme is the consequence of an increased accumulation of the corresponding mRNA and not the consequence of translational or post-translational events. *In situ* hybridization experiments demonstrate that XOR-expressing cells are localized in the epithelial lining of the mammary gland alveoli, but not in the epithelial layer of cells that cover the lactiferous ducts and their main branches. This indicates that the

transcript coding for the enzyme is synthesized in the cells that are responsible for the production and secretion of milk during the *post-partum* lactation phase. The contention is further supported by the fact that the same type of cell is also responsible for the production of β-casein, a prototypic milk protein. *In vivo*, XOR induction follows the surge of lactogenic hormones and the progressive development of the mammary gland alveolar buds, which is observed during the last days of pregnancy. From this moment on, high levels of the enzyme are synthesized until involution of the gland. It is possible that part of the observed elevation in XOR gene expression is due, at least partially, to the increase in the number of secretory epithelial cells, which, in turn, is secondary to the growth and branching of the lactiferous ducts. In fact, low but detectable levels of the XOR transcript are present in the epithelial component of the growing mammary gland well before completion of mammogenesis. However, the dramatic increase in XOR gene expression between days 12 and 18 of gestation is associated with an increase in the amount of XOR transcript present in each mammary epithelial cell. This demonstrates that most of XOR induction is consequent to a *bona fide* increase in the synthesis of the enzyme at the single-cell level upon stimulation with lactogenic hormones. Taken together, our *in vivo* results demonstrate that XOR represents a differentiation marker for the mammary gland secretory alveolar epithelium, and they suggest a potential role for the enzyme in the processes of mammogenesis or lactogenesis.

To study the possible functional significance of XOR expression in the mammary gland and the determinants of the enzyme induction, we turned to the *in vitro* model of mammary epithelial cell differentiation represented by the HC11 cell line. This is an immortalized cell line which has a relatively normal phenotype and responds to a variety of differentiating and lactogenic stimuli with an up-regulation of the genes coding for milk proteins [32]. To respond to lactogenic stimuli, HC11 cells must reach confluency, arrest their growth and start to produce extracellular matrix proteins [34]. Although the mechanisms underlying the phenomenon are obscure, it is known that extracellular matrix proteins are important for the process of mammary epithelial cell differentiation and for the priming of cells to the action of lactogenic hormones [35,36]. In HC11 cells, we observed that XOR is not expressed at detectable levels during the logarithmic phase of growth. As the cells reach confluency, they arrest growth by contact inhibition and start to deposit components of the extracellular matrix and the enzyme is slightly induced. This may just be the result of a reorientation of the nucleotide metabolism towards catabolic pathways, which may be consequent on the proliferation arrest. However, growth-related events do not explain the much more significant induction of XOR when HC11 cells are cultured for a number of days with dexamethasone and prolactin. In this cell line, the hormonal stimuli inducing XOR are similar to those necessary for the expression of milk proteins, such as β-casein [32]. However, a notable difference between the expression of XOR and β-casein (or other milk proteins) is evident. In HC11 cells cultured in 10% (v/v) fetal calf serum + insulin, dexamethasone induces XOR whereas it does not upregulate the expression of β-casein, which strictly requires prolactin in addition to glucocorticoids. Another notable difference between XOR and β-casein expression in HC11 cells is discernible by *in situ* hybridization experiments. While XOR mRNA expression is observed in the whole population of cells in a rather synchronous fashion, β-casein mRNA is evident only in a limited number of cells which increases progressively with time. It is tempting to speculate that the β-casein-positive sub-population may represent the fraction of HC11 cells that underwent complete differentiation towards a secretory phenotype. This suggests that XOR gene expression may precede the expression of β-casein (as well as other milk proteins) and may be associated with an intermediate state of differentiation of the mammary epithelial cell.

The data obtained in HC11 cells strongly suggest that glucocorticoids and prolactin play an important role in regulating the levels of intracellular XOR, and they indicate a role for the two types of hormones in the induction of the enzyme during the late phases of pregnancy and during lactation. As to the molecular mechanisms responsible for the up-regulation of XOR in HC11 cells, this is mainly the result of an elevation in the transcriptional activity of the relevant gene. The transcriptional mechanism is supported by nuclear run-on experiments as well as by the complete inhibition of mRNA induction observed in the presence of dexamethasone or the combination of dexamethasone plus prolactin, when HC11 cells are contemporaneously treated with non-toxic concentrations of the RNA-polymerase II inhibitor, actinomycin D. Further evidence in favour of this mode of action is given by the fact that neither dexamethasone nor dexamethasone plus prolactin significantly alter the half-life of the XOR transcript (M. Kurosaki, S. Zanotta, M. Li Calzi, E. Garattini and M. Terao, unpublished work). At present it is impossible to establish whether the transcriptional activation of the XOR gene is directly modulated by dexamethasone or dexamethasone plus prolactin, or whether it requires the mediation of other *de novo* synthesized proteins, since inhibition of protein synthesis by cycloheximide causes a massive accumulation of the transcript coding for the molybdoflavoprotein. This phenomenon is observed not only following treatment of HC11 cells with the two hormones and the protein synthesis inhibitor, but also in the presence of cycloheximide alone. Accumulation of the XOR transcript following the inhibition of protein synthesis is not specific to mammary epithelial cells, since it is observed also in mouse L929 fibroblastic cells [17], and in the whole animal, at the level of the liver [16]. This suggests that one or more short-lived protein(s) inhibit the transcription of the XOR gene or cause degradation of the corresponding mRNA.

The experiment conducted in the presence of the steroid receptor antagonist RU486 strongly suggests that dexamethasone-dependent induction of XOR is a receptor-mediated process. RU486 inhibition is likely to be the consequence of interaction between the antagonist and the glucocorticoid receptor. In fact, progesterone (10^{-6} M) does not affect the basal level of XOR expression. In addition, the progestative does not alter the induction of XOR by dexamethasone or dexamethasone plus prolactin (M. Kurosaki, S. Zanotta, M. Li Calzi, E. Garattini and M. Terao, unpublished work). However, the long lag-time for the accumulation of the XOR transcript, as well as the lack of corticosteroid responsive element sequences in the promoter region of the gene [1], suggest an indirect regulation by corticosteroids.

Treatment of HC11 cells with the corticosteroid and genistein or erbstatin results in an increase in the XOR transcript which is higher than that observed in the presence of dexamethasone alone. Although the two agents have other biochemical effects besides inhibition of tyrosine kinases, the results are consistent with the fact that one or more members of this class of kinases may negatively regulate the glucocorticoid-dependent regulation of XOR in HC11 cells. The contention is further supported by the fact that inhibition of intracellular phosphatases leads to a block in the up-regulation of the XOR gene caused by dexamethasone and prolactin. Thus, a balance between phosphorylating and dephosphorylating events may control the induced expression of the XOR gene. When HC11 cells are treated with genistein and erbstatin at a concentration that reduces β-casein

expression (Figure 7, and M. Kurosaki, S. Zanotta, M. Li Calzi, E. Garattini and M. Terao, unpublished work), complete inhibition of the synergistic induction of XOR by prolactin and dexamethasone is observed. This is in line with the fact that the prolactin receptor is coupled to the JAK-Stat intracellular pathways [37–41] which involve a cascade of tyrosine phosphorylation events. Stat5 responsive sequences located in the 5′-flanking region of β-casein and other prolactin-regulated genes are similar to the GAS sites which are responsible for the binding of the Stat1-p91 transcription factor activated by type II and type I IFNs [42]. Considering that the XOR gene is responsive to both IFNs and prolactin, it is possible that the gene itself contains a GAS or a GAS-like sequence in its regulatory regions, although a similar structural element is not found in the 5′-flanking region, where the basic promoter elements are located [1].

In conclusion, the data contained in this report demonstrate that the XOR gene product is a biologically significant marker for the differentiation of mammary epithelium and they suggest that it may represent a good experimental model to study the differentiation of the mammary epithelium at the single-gene level.

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