Hormonal regulation of H19 gene expression in prostate epithelial cells

N Berteaux, S Lottin, E Adriaenssens, F Van Coppennolle, X Leroy, J Coll, T Dugimont and J-J Curgy

Abstract

The H19 gene is transcribed in an mRNA-like noncoding RNA. When tumors of various organs or cell types are considered, H19 oncogene or tumor-suppressor status remains controversial. To address the potential regulation of H19 gene expression by an androgen steroid hormone (DHT: dihydrotestosterone) or by a peptidic hormone (PRL: prolactin), we performed experiments in rats systemically treated with chemical mediators. This range of in vivo experiments demonstrated that chronic hyperprolactinemia upregulated the H19 expression in epithelial and stromal cells whereas DHT downregulated the gene. PRL and DHT appeared to be opposite mediators in the H19 RNA synthesis. We investigated these hormonal effects in three human prostate epithelial cell lines. In LNCaP cancer cells, the opposite effect of PRL and DHT was corroborated. However, in normal cells (PNT1A), H19 remained insensitive to the hormones in fetal calf serum (FCS) medium but became responsive in a serum-stripped medium. In the DU-145 cancer cell line, tested for its androgen-independence and aggressiveness, the hormones had no effect on H19 expression whatever the culture conditions. Finally, we demonstrated that PRL upregulated the H19 expression in LNCaP cells by the JAK2–STAT5 transduction pathway. We conclude that H19 expression is regulated by both a peptidic and a male steroid hormone.


Introduction

H19 is an unusual gene transcribed by the RNA polymerase II and processed by capping, splicing and polyadenylation, but all attempts to isolate an H19 protein have so far failed (Pachnis et al. 1988, Brannan et al. 1990). Indeed, a 26 kDa protein has been translated in vitro but only after extensive deletion and/or point mutations in the long 5′-untranslated region of human RNA (Joubel et al. 1996). Consequently, given the evolutionary structure conservation at the RNA level of several mammalian species and the absence of conservation at the protein level, it has been proposed that the mature transcript is the functional product of the H19 gene (Brannan et al. 1990, Juan et al. 2000).

The human H19 gene is an imprinted gene, located on chromosome 11p15·5 and is exclusively expressed from the allele of maternal origin (Glaser et al. 1989, Rainier et al. 1993). An imprinting control region (ICR) is crucial in this imprinting. In vitro and in vivo experiments indicated that CTCF (a DNA-binding zinc-finger protein) mediates the boundary activity of the maternal ICR, and that methylation of the paternal ICR abolishes this activity by preventing CTCF binding. These two elements act as a chromatin boundary, which allows monoallelic H19 expression (Hark et al. 2000, Pant et al. 2003, Schoenherr et al. 2003).

The H19 gene is abundantly expressed in both extraembryonic and fetal tissues and is repressed after birth, except in a few adult organs such as the mammary gland (Douc-Rasy et al. 1993, Dugimont et al. 1995), cardiac and skeletal muscles (Pachnis et al. 1984), the uterus (Ariel et al. 1997, Adriaenssens et al. 1999) and the lung (Poirier et al. 1991). Otherwise, it has been reported that the H19 gene is strongly stimulated under stress conditions (Ayesh et al. 2002, Stuhlmuller et al. 2003).
The possible functional relationship between H19 expression and tumorigenesis is still a matter of debate, as it seems to depend on the organ, the cell type and the cellular environment. Many types of cancer, such as breast (Douc-Rasy et al. 1993, Dugimont et al. 1995, Adriaenssens et al. 1998), bladder (Elkin et al. 1995, Cooper et al. 1996, Ariel et al. 2000), lung (Kondo et al. 1995), esophageal (Hibi et al. 1996) and cervical (Douc-Rasy et al. 1996), carcinomas exhibit H19 overexpression. Importantly, in bladder carcinoma, H19 expression is statistically correlated with tumor grade and is a marker of early recurrence, suggesting that the gene has oncogenic properties (Elkin et al. 1995, Ariel et al. 2000). Furthermore, our group reported that an overexpression of an ectopic H19 gene enhanced the tumorigenic properties of breast cancer cells (Lottin et al. 2002). Other studies have established the tumor-suppressor status of the gene. Indeed, H19 downregulates IGF-2 expression, and it is frequently inactivated in Wilms’ tumor (Moulton et al. 1994, Steenman et al. 1994). In agreement with this, Isfort et al. (1997) reported that the reintroduction of the gene into Syrian hamster embryo cells reduced their tumorigenicity.

In a previous study, we reported H19 overexpression, when compared to healthy tissue, in 72% of breast adenocarcinomas, and this upregulation was significantly correlated with the presence of steroid hormone receptors (Adriaenssens et al. 1998). Later, our group performed in vivo and in vitro experiments that demonstrated that the H19 gene expression was regulated by steroid hormones in both mammary gland and uterus with an up- and down-regulation attributed respectively to 17-β-estradiol and progesterone (Adriaenssens et al. 1999). Collectively, these data prompted us to analyze the H19 expression and its regulation in another organ sensitive to steroid hormones, the prostate.

The organogenesis and secretory activity of the prostate gland are hormonally controlled. It is now clearly established that the growth, differentiation, and programmed cell death of prostate cells are regulated by steroid androgens during both development and adult life (Reiter et al. 1999). For this reason, the main treatment for prostate tumors consists of inhibiting cell growth by suppressing the action or production of endogenous androgens. However, despite this treatment, some tumors, especially malignant ones, can continue to progress. Thus, it has been proposed that nonsteroid factors, such as prolactin (PRL), could also be involved in the proliferation of prostate cells and in the development and regulation of benign hyperplasia of the prostate and prostate cancer (Van Coppenolle et al. 2001).

PRL is a peptidic hormone that exerts pleiotropic biologic effects on a variety of cells and tissues via membrane receptors (Goffin et al. 2002). In particular, PRL has been presumed to have a trophic role required for normal development, growth, organogenesis and secretory activity of the prostate (Steger et al. 1998). Moreover, PRL directly regulates citrate production, one of the major functions of prostate. This regulation is considered to be the reproductive function of PRL in males (Costello & Franklin 1994).

In this work, we studied H19 gene regulation in rats after the systemic action of androgen steroid hormones and of a peptidic hormone, PRL. We showed that, in the lateral lobe of the rat gland, H19 expression was upregulated by PRL, but dihydrotestosterone (DHT) counteracted PRL-mediated enhancement. In the human model, we also confirmed the opposite effects of PRL and DHT in cancerous androgen-dependent cells (LNCaP). Interestingly, PRL has no effect on H19 promoter activity in normal (PNT1A) and in cancerous androgen-independent (DU-145) cells cultivated in 10% FCS medium. Finally, we demonstrated with pharmacologic inhibitors that the upregulation of H19 by PRL occurs by the JAK2–STAT5 transduction pathway.

Materials and Methods

Animals and surgical procedures

Male Wistar rats from Ifsa Credo (L’Avthere, France) were used. The animals were handled in accordance with the European Communities Recommendations for Animal Experimentation. Castration, implantation of Silastic medical-grade silicone tubing (1 cm length, 0·078 cm inside diameter× 0·125 cm outside diameter; Dow Corning, Midland, MI, USA) filled with either testosterone (Sigma) or DHT (Sigma), and hyperprolactinemia induction are described in Van Coppenolle et al. (2001). Chronic hyperprolactinemia was induced in rats by daily injections of sulpiride (40 mg/kg), an antagonist of the type 2-dopamine type receptor that is a mediator of dopamine-induced inhibition of PRL secretion (Debeljuk et al. 1975, Nakagawa et al. 1982). As a consequence, sulpiride enhanced the basal plasma PRL level. The present work has been carried out in the same rats used in the previous study of Van Coppenolle et al. (2001) in which histologic gland alterations have been described.

The lateral lobe was chosen because, of the three rat prostate lobes (dorsal, lateral and ventral), it is considered to be the most hormone-sensitive part of the gland (Schacht et al. 1992, Lane et al. 1997). Furthermore, it has been shown that this lobe gives rise to spontaneous and experimental tumors in response to various hormones, and, finally, it is considered to be the most homologous part to the human prostate (Van Coppenolle et al. 2001).

Cell cultures

PNT1A, a human postpubertal prostate normal cell line immortalized with SV40, was obtained from the ECACC
ISH protocol

Queva et al. (1992) have previously described the basic experiments. After hybridization, slides were dipped into the NTB2 nuclear track emulsion (Kodak) and exposed for 3 weeks. Autoradiographic development (D19 developer) and fixation (Unifix, Kodak) were performed at 12 °C. A fluorescent post-staining of the nuclei was performed (Hoechst, 33258 Bisbenzimide, Serva, λ = 340 nm). Cover slips were fixed with Dako Glycergel (Sebia, Issy les Moulineaux, France). Observations were made through an Olympus BH2 microscope. Labeling levels were evaluated from series of 10–15 sections by slide, each condition including five rats. Medium or high labeling was established independently by three of us versus the weak H19 expression in control rats.

Semi-quantitative RT-PCR

Total RNA was extracted with Tri-Reagent (Euromedex). An amount of 2.5 μg RNAs was treated for 1 h at 25 °C with amplification grade RNase free-DNase I (Invitrogen). Reverse transcription was performed with 60 pmol random hexamers, 50 mM KCl, 10 mM Tris–HCl, 3 mM MgCl2, 0.5 mM dNTP and 20 U RNAse inhibitor, and with murine leukemia virus reverse transcriptase for 45 min at 42 °C. One-twentieth of the cDNA-containing solution was amplified with 1 U Ampli Taq Gold, 25 pmol H19 primers, 1 pmol β-actin primers, 2.5 mM MgCl2, 0.2 mM KCl and 10 mM Tris–HCl (all PCR reagents were provided by Applied Biosystems, Paris, France). For H19 amplification, the primers used were as follows: 5'-CTGTTTCTTTCTCTCTTCAA CGG-3' and 5'-ATGAAGATGGAGTGCCCGGT-3'. For β-actin amplification, the primers used were as follows: 5'-CAGAGAAGAGGCATCCT-3' and 5'-GTGGAAGGTTCCTACATG-3'. The PCR non-saturating conditions were assessed and were as follows: 7 min at 95 °C, then 35 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, and 7 min of final extension at 72 °C. PCR products were run through a 2% agarose gel and visualized with ethidium bromide. The molecular weight (MW) marker used was the PUC19 DNA/MspI marker (MBI Fermentas). Values represent the ratio of the densitometry measurements obtained with the Diversity One program (Pharmacia) for H19 RNA and β-actin mRNA as an internal control. We ensured that RT-PCR experiments were performed in optimal conditions, that is, during the exponential phase of amplification (data not shown). P values were calculated by the t-test, and statistical significance is represented in each figure by asterisks. The meaning of asterisks is as follows: no asterisk: >0.05 not significant, *: 0.01–0.05 significant, **: 0.001–0.01 very significant, ***: <0.001 extremely significant.

Results

H19 expression is enhanced by the induction of chronic hyperprolactinemia in rats systemically treated by sulpiride, and DHT counteracts this effect

We considered that an experimental animal model would be able to define hormone action in prostate cells with a good degree of confidence. We have previously demonstrated that female steroid hormones regulate H19 gene expression, and this analysis has also been assessed in mice systemically treated with female steroid hormones (Adriaenssens et al. 1999). Thus, we sought to discover
Figure 1  H19 gene expression in the rat lateral prostate. ISH with an antisense H19 probe. Animals received systemic treatments, as described in Van Coppennolle et al. (2001). (A) Control; (B) + sulpiride; (C) castrated; (D) castrated + sulpiride; (E) castrated + DHT; (F) castrated + sulpiride + DHT. Scale bar = 200 μm.
whether male steroid hormones could also regulate the H19 gene. We also took this opportunity to test the role of PRL, a peptidic hormone with a major impact on prostate physiology (Costello & Franklin, 1994, Steger et al. 1998). In order to evaluate the control of the H19 gene by steroid or peptidic hormones in the prostate gland, we performed an in vivo study including the systemic action of various treatments. We investigated the effects of hyperprolactinemia alone or in association with androgen delivered by subcutaneous implants containing testosterone or DHT, on H19 expression in the lateral lobe of rat prostate gland. Sections of the lateral lobe of prostate of differentially treated rats were submitted to the ISH technique. In control rats, the basal level of H19 RNA was localized in the both epithelial and mesenchymal cells, but mostly in the former (Fig. 1A). Sulpiride treatment enhanced H19 transcription (Fig. 1A and B). Castration also significantly upregulated the expression of the gene as compared to the control (Fig. 1A and C). Sulpiride injections in castrated rats again strengthened H19 labeling (Fig. 1C and D). Interestingly, this H19 overexpression was accompanied by hyperplasia and chronic inflammation of the gland (Van Coppenolle et al. 2001). DHT treatment alone downregulated the gene expression (Fig. 1C and E), and counteracted H19 activation by PRL, when it was associated with sulpiride injections (Fig. 1D and F). Testosterone produced no obvious reduction of H19 transcription. The results obtained after the various systemic treatments of rats are summarized in Table 1. ISH results were identical whatever the systemic treatments period (30 or 60 days). Only treatments with duration of 30 days are illustrated in Fig. 1. We ensured that the sense probe gave nonsignificant labeling (data not shown).

### Effects of PRL and DHT on H19 gene expression in a normal human epithelial cell line, PNT1A

After the above demonstration of the positive action of PRL on the H19 gene in cancerous LNCaP cells, we checked the effect of this hormone in noncancerous cells. For this purpose, PNT1A cells were cultivated in a medium containing 10% FCS to which 5 nM of PRL were added. Interestingly, in this culture condition, the H19 gene remained insensitive to PRL stimulation contrary to the regulation observed in LNCaP (data not shown). It is noteworthy, too, that PNT1A cells cultivated in a 10% CSS medium yielded similar results to those obtained with LNCaP cells, since in this condition the H19 gene became sensitive to PRL (Fig. 4). Furthermore, as expected from the results obtained with DHT treatments of LNCaP cells (Fig. 3B), we observed that DHT had a negative effect on H19 transcription and suppressed the H19 stimulation by PRL (Fig. 4). Thus, in normal cells cultivated in stripped serum, the opposite effects of PRL and DHT on the H19 transcription were recovered.

### PRL upregulates H19 gene expression by the JAK2–STAT5 transduction pathway

The PRL receptor belongs to the superfamily of hematopoietin or cytokine receptors. Interaction between PRL and its receptor leads to the activation of two major intracellular signaling pathways (Favy et al. 1999). One pathway involves the tyrosine-kinase protein JAK2 (Janus kinase 2) and STAT 5 (signal transducer and activators of transcription) transcription factors. The other consists of a MAP kinase pathway involving the sequential activation of

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**Table 1** Estimation of ISH labeling expressing H19 transcription in the lateral prostate of rats according to the various treatments

<table>
<thead>
<tr>
<th>Treatments</th>
<th>H19 expression in epithelial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Weak</td>
</tr>
<tr>
<td>Sulpiride</td>
<td>Medium</td>
</tr>
<tr>
<td>Castrated</td>
<td>Medium</td>
</tr>
<tr>
<td>Castrated + sulpiride</td>
<td>High</td>
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<tr>
<td>Castrated + DHT</td>
<td>Weak</td>
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<tr>
<td>Castrated + sulpiride + DHT</td>
<td>Weak</td>
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<tr>
<td>Castrated + sulpiride + T</td>
<td>Medium</td>
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**Figure 2** $H19$ gene upregulation by prolactin in LNCaP cells. (A) Cells were grown in a medium containing 10% FCS in absence or in presence of PRL (5 or 50 nM) for 48 or 72 h. After PRL treatment, total RNAs were extracted, and $H19$ expression was evaluated by the semi-quantitative RT-PCR method, with (+RT) or without (–RT) reverse transcriptase. $H_2O$ corresponds to the same PCR performed without cDNA. (B) Values represent the ratio of the densitometry measurements obtained with the Diversity One program (Pharmacia) for $H19$ RNA and β-actin RNA. The latter was used as an internal control. Control conditions are adjusted at 100. Standard deviation was calculated from three independent experiments. Statistical significance is indicated by asterisks, as described in the Materials and Methods section.
Raf, MEK and ERK kinases (Das & Vanderhaar 1996). These two pathways are not exclusive.

To determine the PRL signaling pathway involved in $H19$ activation, we tested different pharmacologic inhibitors. A specific inhibitor of JAK2 kinase (AG490) abolished the $H19$ upregulation by PRL, while a specific MEK1 inhibitor, a critical component of the Raf-ERK/MAPK kinase pathway (U0126), had no effect on the basal $H19$ expression and only slightly reduced the $H19$ stimulation induced by PRL (Fig. 5). To clarify the signal transduction, we tested the effect of two cytokines that act via the JAK–STAT pathway: IL-4, which leads to activation of JAK1/3–STAT6, and GM–CSF, which involves JAK2–STAT5 specifically, like PRL. We failed to obtain any effect on $H19$ expression after these cell treatments (data not shown).

**Discussion**

We have previously reported that in mouse uterus $H19$ expression is stimulated by 17-β-estradiol and inhibited
by progesterone (Adriaenssens et al. 1999). These results prompted us to investigate H19 control by male steroid hormones in the prostate. We also considered a peptidic hormone, PRL, as it has been claimed that this hormone is able to modulate the transcription activity of various hormone, PRL, as it has been claimed that this hormone is not to the hormone, and not to the transduction proteins. Consistently, 50 nM of PRL were less efficient in H19 transcription than the physiological concentration of 5 nM. Moreover, we demonstrated that H19 regulation by PRL is specific to the hormone, and not to the transduction proteins. Interestingly, these in vitro results confirmed the opposite effects of DHT and PRL on H19 gene activity in prostate cells of rat systemically treated with these hormones.

Figure 4 PRL and DHT effects on H19 expression in a normal prostate cell line (PNT1A). PNT1A cells were grown in a medium containing 10% stripped serum without PRL (control), with PRL (5 nM) or with DHT (10\(^{-8}\) M) alone or in presence of PRL (5 nM) for 72 h. After treatment, total RNA was extracted, and the amount of H19 transcripts was evaluated by the semiquantitative RT-PCR method. Values represent the ratio of the densitometry measurements, obtained with the Diversity One program (Pharmacia), for H19 RNA and β-actin RNA. The latter was used as an internal control. Control conditions were adjusted at 100. Standard deviation was calculated from three independent experiments. Statistical significance is indicated by asterisks, as described in the Materials and Methods section.

To ascertain the role of hormones in H19 gene expression in prostate, we first performed an in vitro study of the systemic action of chemical and hormonal mediators in rats. ISH data clearly demonstrated that PRL upregulated H19 expression and DHT counteracted this activation. Nevertheless, testosterone did not alter the H19 expression. Consistently, testosterone is converted to DHT by 5-alpha-reductase in prostate (Schroder 1994, Wang et al. 2004) and consequently, DHT is more active than testosterone during prostate growth (Van Coppenolle et al. 2001).

We wondered whether H19 transcription could be regulated by the hormones mentioned above in human prostate epithelial cell lines. We decided to use the semiquantitative RT-PCR technique for the following experiments of this work. The chosen cancerous androgen-dependent cells (LNCaP) were sensitive to PRL, inducing H19 upregulation. This activation is reminiscent of the PRL-positive action in epithelial prostate cells from systemically sulpiride-injected rats. Two PRL concentrations were tested. For many cytokines that induce receptor dimerization, a high dose of PRL is less efficient than a low one (Fuh & Wells 1995). Consistently, 50 nM of PRL were more efficient in H19 transcription than the physiological concentration of 5 nM. Moreover, we demonstrated that H19 regulation by PRL is specific to the hormone, and not to the transduction proteins. Interestingly, these in vitro results confirmed the opposite effects of DHT and PRL on H19 gene activity in prostate cells of rat systemically treated with these hormones.

In normal prostate epithelial cells (PNT1A) cultured in 10% FCS medium, PRL had no effect on H19 expression. In contrast, when these cells were cultured in a medium containing 10% of stripped FCS, PRL activity on H19 and the opposition between PRL and DHT functions on the gene were recovered.

After having studied the biologic effect of PRL on H19 in normal or androgen-dependent cancerous cells, we investigated H19 regulation in an androgen-independent cancerous line. A human cancerous cell line of high grade (DU-145) was chosen to perform the following strategy. It is noteworthy that H19 seemed to be completely repressed in these cells, as the RT-PCR technique was unable to detect any H19 RNA. Furthermore, the potential upregulation of the gene by PRL was no longer found. Oddly, PRL is able to induce DU-145 cell proliferation, whereas, as expected, DHT had no proliferating effect on these cells (Janssen et al. 1996). Conversely, these authors reported that although DHT markedly stimulated LNCaP proliferation, PRL exerted only a faint, if any, effect on their proliferation. In conclusion, it seems interesting to note that in androgen-dependent cells (LNCaP), PRL and DHT exhibited different effects on their proliferation and induced opposite H19 expression regulation.

Figure 5 PRL signaling pathway involved in the H19 gene activation. Cells were grown in medium containing 10% FCS. Pharmacologic inhibitors specific to the JAK2 kinase/STAT5 (AG490) or to the Raf-ERK/MAPK kinase (U0126) were used independently or in association with 5 nM PRL. After treatment, total RNA was extracted, and the amount of H19 transcripts was evaluated by the semiquantitative RT-PCR method. Values represent the ratio of the densitometry measurements, obtained with the Diversity One program (Pharmacia), for H19 RNA and β-actin RNA. The latter was used as an internal control. Control conditions were adjusted at 100. Standard deviation was calculated from three independent experiments. Statistical significance is indicated by asterisks, as described in the Materials and Methods section.

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It would appear that correlation between PRL action and prostate carcinoma occurrence in men is a matter of debate. The PRL receptors are detected in benign prostate hyperplasia and highly synthesized in dysplasia, but only slightly in higher-grade carcinomas (Leav et al. 1999). A high blood concentration of PRL in some prostate cancer patients has been reported (Harper et al. 1976, Horti et al. 1998). Although Stat tin et al. (2001) have established only a slight correlation between hyperprolactinemia and increased risk of prostate cancer in men, Ben-Jonathan et al. (2002) recently reported that PRL increases prostate weight and promotes development of dysplasia and adenocarcinoma of the dorsolateral lobe of the rat prostate. The latter group suggested that PRL might contribute to early carcinogenesis by activating several target genes. Our study suggests that the H19 gene could be included among these target genes. In addition, data obtained from the androgen-independent cell line (DU-145) could also mean that H19 is no longer dependent on hormonal controls in aggressive cancer cells.

The literature dealing with H19 expression modulation by various biologic factors mentions that H19 regulation results in a delicate equilibrium (Lottin et al. 2003). Here, we report an illustration of this balance through the demonstration that peptidic and androgen-steroid hormones exerted opposite control over H19 gene expression.

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