H19 mRNA-like Noncoding RNA Promotes Breast Cancer Cell Proliferation through Positive Control by E2F1*

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The imprinted H19 gene has riboregulatory functions. We show here that H19 transcription is up-regulated during the S-phase of growth-stimulated cells and that the H19 promoter is activated by E2F1 in breast cancer cells. H19 repression by pRb and E2F6 confirms the E2F1-dependent control of the H19 promoter. Consistently, we demonstrate by chromatin immunoprecipitation assays that endogenous E2F1 is recruited to the H19 promoter in vivo. The functionality of E2F promoter sites was further confirmed by gel shift and mutagenesis experiments, revealing that these sites are required for binding and promoter response to E2F1 exogenous expression and serum stimulation. Furthermore, we show that H19 overexpression confers a growth advantage on breast cancer cells released from growth arrest as well as in asynchronously growing cells. The H19 knockdown by small interfering RNA duplexes impedes S-phase entry in both wild-type and stably H19-transfected cells. Based on these findings, we conclude that the H19 RNA is actively linked to E2F1 to promote cell cycle progression of breast cancer cells. This clearly supports the H19 oncogenic function in breast tumor genesis.

The H19 gene is one of the first genes to have been proven to be imprinted. It is located on chromosome 11 p 15.5 and lies within 200 kbp downstream of the IGF-2 gene (1). These two genes are imprinted in opposite directions, so that the paternal H19 allele is selectively expressed (2, 3). H19 encodes a spliced and polyadenylated RNA that lacks conserved open reading frames but does have a conserved secondary RNA structure (4). Extensive deletions and/or point mutations in the 5′-untranslated region of an ectopic human H19 RNA enable 26-kDa protein translation (5), but no endogenous translation product has so far been identified (6, 7). Therefore, it was quickly proposed that H19 RNA functions as a riboregulator (8).

H19 expression is developmentally regulated. It is abundantly expressed in both extraembryonic and fetal tissues and is repressed after birth except in a few adult organs, particularly in the mammary gland (9, 10). Since the first mention of H19 in 1984 by Pachnis et al. (6), its functions have only begun to emerge. It has been reported that H19 RNA was involved in the repression of the IGF-2 oncogene by affecting its transcription (11) or its translation (12). Recently, we brought evidence that the H19 gene post-transcriptionally up-regulates the thioredoxin, a key protein of the cellular redox metabolism (13).

Despite interesting new insights, the status of the H19 gene in cancer is still a matter of debate. It has been suggested that H19 functions as a tumor suppressor in some Wilms’ tumors, embryonic rhabdomyosarcoma, and the Beckwith-Wiedmann cancer predisposing syndrome (14–16). Consistently with this function, some studies conclude that it down-regulates the IGF-2 factor (11). By contrast, other studies including ours suggest that H19 may play a key role in tumorigenesis and could match the cell aggressiveness (17, 18).

H19 activation has also been reported in various cancer tissues: breast (9, 10, 19), bladder (20, 21), lung (22), and esophageal cancers (23). Its oncogenic role has been well documented in the bladder, since it is considered as an oncodevelopmental marker (24) and regulates genes involved in metastasis and blood vessel development (25). These observations support a H19 role in tumor invasion and angiogenesis. In breast cancer, the oncogenic role of H19 has been well established (26), but the precise gene function in cellular processes is not yet understood. Furthermore, H19 promoter regulation remains widely undetermined. Only a few studies have reported H19 expression to be modulated by cytokines in cells (27–30). However, despite putative regulatory sequences localized in the H19 promoter and the identification of potential transacting factors, no direct regulation of the H19 promoter has so far been described. In a previous work, we reported a negative regulation of H19 promoter activity by the tumor suppressor p53 that would be mediated by protein/protein interactions (31). Consistently, Sorensen et al. (32) proposed a functional interaction between p53 and DP1 (an E2F transcriptional partner) to explain the loss of the dihydrofolate reductase promoter activation by E2F1 in SAOS-2 cells. Because of the presence of two putative E2F consensus sites in the H19 minimal promoter, we hypothesized a similar mechanism.
for H19 promoter control, and we anticipated a role for E2F1 in the regulation of H19 promoter activity. Consequently, we have further investigated the H19 physiological function through this regulation, and we have logically focused on cell cycle progression. E2F transcription factors consist of a related protein family that includes seven distinct E2F members, which act on gene promoters regulated during the cell cycle (33, 34). The first member of the E2F family to be cloned, E2F1, is considered to be the critical factor in G1/S transition (35). E2F1 has been shown to be involved in the transcriptional regulation of several genes, whose products participate in cell cycle progression and DNA synthesis and whose expression is up-regulated at the cell cycle G1/S transition (36–38). We focused our work on E2F1 protein, which does have biological consequences in carcinogenesis. Indeed, E2F1 enhances neoplasia in the skin of transgenic mice and causes tumor formation in the liver (39, 40). Furthermore, E2F1 is involved in breast cancer development through transcriptional activation of the breast cancer susceptibility gene BRCA1 (41) and serves as the primary link between proliferation control and apoptosis (42).

The purpose of the present study was thus to investigate the role of H19 in the cell cycle via a regulation by E2F1. We report that the cell cycle-dependent regulation of the H19 gene is primarily controlled by E2F1 through binding to H19 promoter.

pRB and E2F6, two E2F-dependent transcription inhibitors, repress H19 gene expression. We further focused our study on the biological effect of the H19 gene on cell cycle progression and particularly at the G1/S transition. We show that H19 overexpression confers an obvious growth advantage in breast cancer cells released from growth arrest as well as in asynchronously growing cells. In addition, the knock-down of H19 expression by small interfering RNA (siRNA) impedes progression through the S-phase of the cell cycle. In conclusion, we elucidate the involvement of the H19 mRNA-like noncoding RNA in cancer cell proliferation. Our results clearly shed light on the oncogenic status of H19 in breast cancer cells.

MATERIALS AND METHODS

Cell Culture and Breast Tissues—The human breast cancer epithelial cell lines were obtained from the ATCC and maintained routinely in MEM, containing 5% fetal calf serum (FCS). MCF-7 and T47D are estrogen-sensitive cell lines. BT20 and MDA-MB-231 are estrogen-insensitive cell lines. Normal breast epithelial cells come from primary culture of normal breast tissue resections obtained from modeling surgery. The highly tumorigenic MCF-7Ras cell line corresponds to the H-Ras-transfected MCF-7 cell line. Human embryonic kidney cells (HEK 293) were provided by Dr. D’Halluin (Institut de Recherche sur le Cancer de Lille, France) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS. Cells were harvested with a reporter lysis buffer (Applied Biosystems) 24 h (for MCF-7 cells) and 48 h (for HEK cells) after transfection. Luciferase and β-galactosidase activities were measured by using an AB luciferase assay kit and a β-galactosidase assay kit, as specified by the manufacturer, with a Lumat LB 9501 luminometer (Berthold).

RNA Interference—RNA interference was carried out by using synthetic siRNA duplexes, as described by Elbashir et al. (43). Two synthetic siRNA duplexes (siH19A and siH19B), corresponding to the H19 mRNA sequence 5′-CCACACACUGAUGAGAAUUA-3′ and 5′-GCUA- GAGAACAAGAGCUCU-3′, respectively, were used to inhibit H19 mRNA expression. A synthetic siRNA duplex (siE2F) corresponding to a predicted E2F1 mRNA sequence 5′-GGAGGAAGAGAGGCCUC-3′ was used as a negative control. The siRNA duplexes were purchased from Eurogentec. Cells were grown on coverslips in 6-well plates and transfected with 100 nM of siRNAs (NEXTEC of Eurogentec, as recommended by the manufacturer). To monitor the transfection efficiency, a tagged siRNA duplex was transfected in parallel, and the transfection rate was evaluated by FACs and seen to correspond to 80–90% of transfected cells. After transfection, cells were lysed for total RNA isolation or counted for growth assays.

Northern Blot Analysis—Total RNA was extracted using the guanidium isothiocyanate-CsCl gradient method (44). RNA (20 μg) was denatured, electrophoresed through a 1.2% agarose gel containing formaldehyde, and transferred by capillarity onto a nitrocellulose membrane (Hybond-C-extra; Amersham Biosciences). The H19 mRNA-like noncoding mRNA sequence 5′-GCGUGAGCCUGAGGUCUACU-3′ was used as a control. The siRNA duplexes were purchased from Eurogentec. Cells were grown on coverslips in 6-well plates and transfected with 100 nM of siRNAs (NEXTEC of Eurogentec, as recommended by the manufacturer). To monitor the transfection efficiency, a tagged siRNA duplex was transfected in parallel, and the transfection rate was evaluated by FACs and seen to correspond to 80–90% of transfected cells. After transfection, cells were lysed for total RNA isolation or counted for growth assays.

Real Time RT-PCR—Total RNA was isolated using the Nucleospin RNAII isolation kit (Macherey-Nagel, Hoerdt, Belgium). Reverse transcription was performed with 1 μg of RNAs, 1 μg of random hexamers, 2.5 μg of Moloney marmoset leukemia virus reverse transcriptase, and 0.1 units of reverse transcriptase (Invitrogen) for 1 h at 37 °C in a final volume of 100 μl. Real time PCR amplifications were performed using a Quantitect SYBR®Green PCR kit (Qiagen, Courtaboeuf, France) with 2 μl of cDNA and 500 nM of primers. The primers used were as follows: 5′-GGAGTAGGATGAGCTCAGG-3′ and 5′-CTAAGGGTTGCAAGGAGGCT-3′ for the H19 transcript, 5′-TTACTCTCCACAGGAGTTC-3′ and 5′-CTGAGTAGATGGAGCACGTCG-3′ for H19 near the siH19A; 5′-GGCTTTTATCCGGCAGC-
H19 Promotes Cell Proliferation

**RESULTS**

**H19 Expression Is Transcriptionally Up-regulated during the S-phase of Cells Stimulated to Enter the Cell Cycle**—Cell growth-dependent control of the E2F1 gene and E2F1-regulated genes has been described (37, 49, 50). We previously showed down-regulation of H19 by p53 and anticipated a possible role for p53 in E2F1 activity repression (31). These indications led us to examine whether H19 expression was cell cycle-dependent in MCF-7 cells. Cells were serum-starved and subsequently induced to reenter the cell cycle by serum addition. Cells were harvested after starvation or at various times following serum addition. Fig. 1A shows the FACS analysis of these cells, indicating that most of them are in the S-phase after 32 h of stimulation. Cells treated under these various conditions were checked for expression levels of both H19 and E2F1 RNAs. Expression levels of both genes were low in serum-starved cells (Fig. 1B). As expected, E2F1 transcription was up-regulated by serum addition, with a peak at the G1/S-phase boundary (16–20 h). In parallel, H19 mRNA synthesis was activated and reached a high expression level at 32 h, coinciding with the greatest number of cells in S-phase. Thus, both genes are transcriptionally activated after serum addition, although the timing differs, since the target H19 gene response takes longer than the E2F1 induction. Collectively, these results show the cell cycle-regulated expression of the H19 gene and suggest that H19 is likely to be regulated by E2F1.

**E2F1 Activates the H19 Promoter**—The H19 gene possesses a TATA-less promoter, a feature reminiscent of many known

**Western Blot—Nontransfected or RNAi-transfected cells were harvested in lysis buffer. 30 μg lysates were subjected to SDS-PAGE, transferred onto a nitrocellulose membrane (Invitrogen; Millipore Corp., Billerica, MA), and exposed to autoradiography. The reaction was revealed using the chemiluminescence kit ECL (Amersham Biosciences) with Eastman Kodak Co. hyperfilm.**

**Fluorescent Analysis and Cell Sorting (FACS)—Cells from a 10-cm dish were trypsinized, centrifuged, washed with PBS, and fixed in 70% ethanol. For transfected cells, selection was made with the cotransfection of a GFP expression plasmid. In this case, cells were previously fixed in paraformaldehyde for 30 min at 4 °C, washed twice with PBS, and subsequently fixed in 70% ethanol for 30 min at 4 °C. For propidium iodine (PI) staining, cells were washed twice in PBS, centrifuged, and resuspended in 500 μl of PI buffer (500 μl of PBS, 100 μg of PI, and 100 μg of RNase A). After 30 min of incubation at 37 °C, the samples were analyzed with a Becton Dickinson FACScan.**

**Chromatin Immunoprecipitation Assays—Detection of promoter-bound E2F1 proteins was assayed by chromatin immunoprecipitation assays, essentially as previously described (47, 48). Cells were treated with formaldehyde to form cross-links between E2F and associated promoter regions. Chromatin was then isolated, fragmented by sonication, and subjected to immunoprecipitation by using antibody directed against E2F1 (catalog number SC-193; Santa Cruz Biotechnology). An antibody against the FLAG epitope (catalog number SC-807; Santa Cruz Biotechnology) was used as a negative control (nonrelevant antibody). To detect the H19 gene in protein-DNA complexes, a 141-bp fragment located in the promoter was amplified by PCR using oligonucleotides 5'-GTCTGGGAGGGAGAGGTCCT-3' and 5'-CCACTCTCTCTGGCAGAACG-3'. The primers used to detect the E2F1 gene promoter were used by others (46). The two genes were detected by PCR with specific primers corresponding to the wild-type or E2F-mutated site in the promoter regions. Chromatin was then isolated, fragmented by sonication, and subjected to immunoprecipitation by using antibody directed against E2F1 (catalog number SC-193; Santa Cruz Biotechnology). An antibody against the FLAG epitope (catalog number SC-807; Santa Cruz Biotechnology) was used as a negative control (nonrelevant antibody).**

**Electrophoretic Mobility Shift Assay—Double-stranded oligonucleotides were generated corresponding to the wild-type or E2F-mutated site II of the H19 promoter. The wild-type oligonucleotide was end-labeled for use as a probe. The E2F binding site is underlined, and the mutated nucleotides are shown in boldface type: H19WT, 5'-GAATTCTGGCGGGCCACCCCA-3'; H19mut, 5'-GAATTCTGGGAGGGAGAGGTCCT-3'. These double-stranded oligonucleotides were also used as competitors at a 400-fold molar excess. As a positive control, we also performed an electrophoretic mobility shift assay with a typical E2F site from Nushift™ Kit (Geneka Biotechnology, Inc., Strasbourg, France). For binding experiments, the following components were mixed and preincubated at room temperature for 15 min and depending on the experiment: 4 μl of complete cell extracts of COS7 cells transiently both transfected and untransfected by E2F1 vector, 4 μg of competitors, 4 μl of E2F1 mononuclear antibody (Nushift™ kit), 10 μl of binding buffer (500 ng of sheared salmon sperm DNA, 25 mM Hepes, pH 7.6, 25 mM KCl, 1 mM EDTA, 2 mM MgCl₂, 0.05% Nonidet P-40, 10% glycerol) in a final assay volume of 20 μl. Following preincubation, the labeled oligonucleotide was added (2.5 ng), and the mixtures were incubated for another 15 min at room temperature. Samples were loaded onto a 4% nondenaturing polyacrylamide gel in TBE (22.5 mM Tris borate, 0.5 mM EDTA) at 4 °C. The gel was dried, and autoradiography was performed.**

**FIG. 1. Cell cycle-regulated expression of H19 mRNA. A, time course cell cycle distribution of MCF-7 cells. MCF-7 cells were serum-starved and stimulated to enter the cell cycle by the addition of 10% serum-containing medium. The cell cycle distribution was analyzed by FACS at the indicated times after induction. B, analysis of the H19 mRNA expression in MCF-7 cells during quiescence and serum stimulation. Cells were treated as described for A. At the indicated times after serum addition, H19 mRNA was analyzed by Northern blotting. Blots were probed for human H19 and E2F1 mRNA (upper blots) and subsequently probed for ARPP0 (acid ribosomal phosphoprotein 0) expression as an internal control (lower blot).**
E2F-responsive genes. Moreover, a screening of the H19 promoter sequences revealed two putative E2F recognition sites in proximity to the H19 transcription start site (sites I and II boxed in Fig. 4A). These properties, together with the finding that H19 expression was cell cycle-regulated, prompted us to examine whether the H19 promoter was directly regulated by E2F1. We tested this hypothesis by performing transient transfections with a luciferase reporter vector under the control of the minimal H19 promoter (31). This reporter vector was co-transfected into MCF-7 cells together with a plasmid-expressing E2F1 wild-type protein (E2F1), or an E2F1 version deleted in its transactivation domain (E2F1Δ). E2F1 expression in

**Fig. 2. H19 promoter activation by E2F1.** A, MCF-7 cells were transfected with 1.2 μg of H19-luciferase reporter gene and 0.5 μg of pCMV-E2F1 or pCMV-E2F1Δ expression plasmids. Parental pCMV vector was used as controls. The Cdc6-luciferase gene was used as a positive functional control. Transfected cells were cultured in medium with 5% FCS for 24 h. B, inhibition of E2F1 expression by specific siRNAs. MCF-7 cells were transfected with 400 pmol of siE2F1 or siGFP used as control conditions, and proteins were processed for Western blotting using an anti-E2F1 antibody. C, knock-down of E2F1 decreases H19 expression. MCF-7 cells were transfected with the E2F1-specific siRNA (siE2F1) or with the control siRNA targeting the green fluorescent protein (siGFP). Endogenous H19 expression was measured by real time PCR and expressed in relative H19 expression with regard to the RPLPO reference gene, as described under “Materials and Methods.” E2F6 (D) and pRb (E) act as negative regulators of H19 promoter. MCF-7 cells were transfected with 1.2 μg of H19-luciferase reporter gene and 0.5 μg of expression vectors (pCMV-E2F6 and pSG5-Rb and/or pCMV-E2F1). Empty pCMV and pSG5 vectors were used as controls. Transfected cells were cultured in medium containing 5% FCS for 24 h. For all relative luciferase activities, pCMV-β-galactosidase (0.2 μg) was cotransfected as an internal control. Luciferase and β-galactosidase activities of cell extracts were measured. Luciferase activity of cell extracts was normalized to β-galactosidase activity and is plotted for each transfected empty vector. All values shown represent the mean values of three independent experiments.
MCF-7 cells induced a 5-fold activation of the H19 promoter 24 h after transfection, whereas E2F1α severely reduced H19 promoter activity (about 25% remaining activity compared with the control basal level) (Fig. 2A). The latter feature was probably due to the competition of E2F1α, considered to be a negative transdominant mutant, with the endogenous E2F1 wild-type proteins. We obtained similar results in two unrelated cell lines (HeLa and HEK-293 cells) treated following the same procedure (data not shown). In parallel, as a functional control in MCF-7 cells, we checked E2F1 plasmid activity by using a known E2F1-regulated gene (i.e., the Cdc6 gene) (49). As expected, this gene was obviously up-regulated by E2F1, the phenomenon reaching about a 12-fold increase.

Then we investigated the effect of E2F1 inhibition by RNA interference on the endogenous H19 expression in MCF-7 cells. When the E2F1 protein level is decreased by siRNA targeting human E2F1 mRNA (Fig. 2B), H19 expression measured by real time RT-PCR is strongly reduced with regard to the negative control siRNA targeting green fluorescent protein (siGFP) (Fig. 2C). Finally, we tested the effect of two E2F1-negative regulators: E2F6, which lacks the transactivation domain (51, 52), and pRb, which complexes and inactivates E2F1. As expected, E2F6 repressed the H19 basal expression by about 50% in transiently transfected MCF-7 cells (Fig. 2D). Results shown in Fig. 2E indicate that pRb had only a weak direct negative effect on H19 promoter activity in these cells but was able to completely suppress the H19 transactivation by E2F1. This suggests that pRb indirectly represses the H19 promoter through the inhibition of E2F1 activity. Taken together, these results confirm the E2F1 involvement in H19 promoter regulation.

**In Vitro and in Vivo Association of E2F Proteins with the H19 Gene**—As mentioned above, we found two sequences in the H19 promoter (I and II) matching the consensus of an E2F binding site. The functionality of the E2F sites was investigated by electrophoretic mobility shift assays using wild-type and mutated probes. We chose a radiolabeled probe, which was a sequence of 21 nucleotides overlapping the site II (Fig. 3A).

**FIG. 3. In vitro and in vivo E2F1 binding to the H19 promoter. A**, nucleotide sequences of the WT or mutated H19 probes used in gel mobility shift assays. **B**, gel mobility shift assays were performed with COS-7 cell extracts and the end-labeled E2F fragment from the H19 promoter containing the wild-type site II as a probe. Lane 1, the probe only (probe); lane 2, the probe and the non-transfected cell extracts (NTCE); lane 3, the probe and the E2F1-transfected cell extracts (TCE). Competition experiments were performed by adding a 400-fold molar excess of unlabeled oligonucleotides to the gel shift reaction mixtures. The competitor oligonucleotides used were the wild-type site II (lane 4) and a mutant form of this sequence (lane 5). As a positive control, we also competed with a typical E2F site from the Nushift™ kit (lane 6) and a mutant form of this sequence (lane 7). A supershift was obtained when an antibody directed against E2F1 was added to cell extract (lane 8). **C**, immunoprecipitation of E2F-associated H19 promoter fragment. HeLa cells were treated with formaldehyde to create cross-links between transcription factors and chromatin. The chromatin was isolated, sheared, and immunoprecipitated using antibody directed against E2F1 or nonrelevant antibody (NR Ab). The presence of chromatin fragments corresponding to the H19 gene or to the E2F1 gene promoter was assessed by semiquantitative PCR using gene-specific primers. Recovery of H19 and E2F1 gene fragments from the protein-DNA extracts (prior to the immunoprecipitation) is shown in lane 1. Negative control primers correspond to primers located out of the H19 promoter (at +40,510 bp to the H19 transcription start site). Weak amplifications, appearing only after 42 cycles of PCR in immunoprecipitations with nonrelevant and α-E2F1 antibodies, are nonrelevant. The PCRs were separated by electrophoresis on a 2% agarose gel.
sequences of E2F recognition sites in wild-type (H19WT) and mutated (Mut-I, Mut-II, and DMut) constructs. Site I sequences are promoter (H19WT) or the mutated H19 activities like in Fig. 2. All values shown represent the mean values of three independent experiments. Transfected cells were cultured in medium containing 5% FCS for 24 h. Luciferase activities were normalized to TCE.

In a more physiological context, using the in situ H19 promoter, we tested if E2F proteins associate with these regulatory sequences in vivo by chromatin immunoprecipitation experiments (Fig. 3C). Chromatin was subjected to immunoprecipitation by using antibody directed against E2F1. This led to a supershift of the complex (Fig. 3B, lane 8). Thus, E2F1 can be seen to bind directly to the H19 promoter throughout the site tested.

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mRNA and E2F1 proteins (Fig. 6, We studied the expression levels of E2F1 Epithelial Cells—transcriptional activation during growth stimulation. Serum stimulation (Fig. 5 A) of cells, as well as in cells stimulated to reenter the cell cycle (Fig. 7B). These results indicate that a H19 overexpression gives a new phenotype to cells, since it favors their S-phase entry after serum stimulation. To test this hypothesis, we used H19-overexpressing cells. We previously characterized MDA-MB-231 cells, which contain a very low level of endogenous H19 RNA and were stably transfected with human H19 genomic sequences under the control of a heterologous promoter (26). These clones, which expressed a bona fide H19 RNA, were checked for H19 overexpression by real time RT-PCR. After normalization, S14-3 and S14-4-H19-transfected clones exhibited an H19 overexpression of 35- and 70-fold, respectively, when compared with mock-transfected cells (Neo) (Fig. 7, A and B). We investigated the effect of this H19 overexpression on the S-phase entry of these cells. Cells were starved and subsequently induced to reenter the cell cycle by serum addition. We measured the cell percentage in the S-phase by FACS analysis. At 18–24 h, the S-phase percentage of H19-overexpressing cells (S14-3 and S14-4) was significantly higher than the percentage of the control cells (Neo) (Fig. 7C). In parallel, we monitored the H19 effect on cell growth. H19-transfected and Neo cells were plated (T0), serum-starved, and grown in a 10% FCS-containing medium for 48 h (T48). S14-3 and S14-4 clones displayed nearly twice as many cells as control cells (Fig. 7D). These results indicate that a H19 overexpression gives a new phenotype to cells, since it favors their S-phase entry after serum stimulation and confers an obvious advantage for cell proliferation.

Our studies have shown that H19 is maximally expressed during the S-phase and that the H19 overexpression accelerates S-phase entry in cells entering the cell cycle from quiescence (Figs. 1 and 7, C and D). These results are consistent with the proposal that H19 plays a significant role in cell cycle progression. However, it remains to be determined whether H19 promotes cell cycle progression in asynchronously growing recombinant cells expressing ectopic H19. To address this point, we decided to specifically silence H19 expression in these cells by carrying out RNA interference assays. We tested two siRNA duplexes targeting H19 RNA and monitored the decrease in the H19 RNA level by real time PCR. As illustrated for the S14-4 clone (Fig. 8, A and B), transfection of these sequences reduced H19 RNA level by about 75% for siH19A.
and 80% for siH19B compared with the control siGFP. We obtained the maximal inhibition (90%) by using a mixture of the two sequences, and consequently, this condition was applied for the following assays. To examine the specific effect of H19 expression on cell cycle progression, we performed growth assays with the asynchronously growing cells Neo, S14-3, and S14-4 and these latter two clones transfected with siRNA targeting either H19 or GFP as a negative control. As shown in Fig. 8C, H19 overexpression conferred a growth advantage on asynchronous cells (compare Neo and S14-4). In addition, siRNA that specifically targeted H19 sequences resulted in a significant decrease in cell growth compared with the control siRNA (compare S14-4siH19 and S14-4siGFP). We confirmed that H19 knock-down by siRNA was still efficient 72 h post-transfection. We obtained similar results with the other H19-overexpressing clone (S14-3) (data not shown). Collectively, these results demonstrate that H19 is able to promote cell growth in breast cancer cells.

The inhibition of cell growth by the H19-specific siRNAs could result from a S-phase entry block or from an inhibition to progression through other phases of the cell cycle. In order to distinguish between these possibilities, we examined the cell cycle distribution of cells treated with H19-specific siRNA by FACS analysis in serum-starved cells. The siH19-treated cells were distributed over all phases of the cell cycle, with an accumulation in G0/G1-phase, since the percentage of cells is increased from 37 to 51% (Fig. 8D). In parallel, we performed growth assays using cell counting, and we obtained about a 20% decrease in the cell number after a 24-h starvation period, when cells were transfected with siH19 compared with the siGFP-transfected control cells (data not shown). Finally, we tested the effect of the endogenous H19 knock-down in MCF-7 cells to evaluate the physiological contribution of the gene to cell proliferation (Fig. 8E). RNA interference on H19 gene reduces the cell number by about 10% after 5 days of culture compared with the control siGFP sequences. Thus, our results demonstrate that the H19 mRNA-like noncoding RNA plays a significant function in favoring S-phase entry.

**DISCUSSION**

H19 is transcribed in an untranslated RNA molecule (8), which accumulates in the human placenta and several fetal tissues, and probably plays a pivotal role in embryogenesis and fetal growth and development (59). In addition, overexpression or reexpression of the gene occurring in numerous human cancers indicate that H19 is involved in oncogenesis (17). Several lines of evidence presented in this study show that H19 is a bona fide E2F target gene and promotes cell cycle progression.

We report the cell cycle-dependent expression of the H19 gene in breast cancer cells. We demonstrated by chromatin immunoprecipitation assays that endogenous E2F1 was recruited to the H19 promoter in vivo and that E2F site mutation prevented complex formation in in vitro band shift experiments. These results conclusively show that a specific and direct E2F1 binding to E2F consensus sites can occur in the human H19 promoter. Transient transfection experiments in MCF-7 cells demonstrated that the H19 promoter is activated by E2F1 proteins through two
E2F recognition sequences present in the promoter. In functional assays, disruption by point mutation of E2F binding sites in the H19 promoter abolished the E2F1 ability to up-regulate gene transcription, confirming the potent role of both E2F recognition sequences. Thus, the E2F sites within the 5' regulatory region of the H19 gene are essential for gene induction. Moreover, we observed a H19 promoter repression by E2F1, which may compete with endogenous wild-type proteins through its transdominant negative properties. In addition, E2F1 activity is required for endogenous H19 expression, as shown by RNA interference assays.

The H19 promoter was sensitive to another E2F member, E2F6, considered to be a transcriptional repressor, since it lacks both transactivation and pocket protein-binding domains. E2F6 was able to impede the endogenous E2F complex activity on the H19 promoter. This inhibition can be mediated either by exclusion of other E2F family members from the DNA, or through the recruitment of cellular factors that actively inhibit transcription (51, 52). Furthermore, it is widely accepted that E2F family binding to pRb family proteins (pRb and related pocket proteins p107 and p130) is the primary regulatory mechanism for E2F family activity. For instance, E2F1 binds almost exclusively to the pRb member. The association between the two protein types forms inactive complexes, hence inhibiting E2F-dependent transcription (37, 60). In agreement with these results, transfection in MCF-7 cells of a pRb expression vector abolished the H19 gene transactivation by E2F1. This finding brings supplementary proof of the H19 promoter control by E2F1.

The above results have clearly established the cell cycle-dependent expression of H19, and we further investigated the role of E2F sites present in the H19 promoter in this regulation. Indeed, mutations of these sites abolished the activation of H19 expression in S-phase during growth stimulation, clearly indicating the E2F1 control of H19 gene transcription during the cell cycle. Interestingly, the E2F1 gene, like H19, is broadly but not uniformly expressed during mouse embryogenesis (61) and is often deregulated in human cancers (42).

We therefore investigated the H19 and E2F1 expression patterns in various breast epithelial cells. The study revealed a correlation between H19 and E2F1 expression levels. Both genes were weakly expressed in normal breast cells and up-regulated in breast cancer cells. We also used the isogenic model MCF-7/Ras. MCF-7/Ras cells, when stably transfected with the H-Ras oncogene exhibit high tumorigenic properties. This cell line lost its estrogen dependence, secreted diffusible growth factors that support its own tumor growth in vivo, and displayed an increased invasion capacity in vitro assays (62, 63). It is interesting to note that H19 and E2F1 expressions were activated in these cells when compared with the parental MCF-7 cells. This suggests that H19 and E2F1 expression levels match with the cancer cell aggressiveness. MDA-MB-231 cells are the only exception, where the expression of the two genes were dissociated. These cells exhibit a very low level of H19 transcripts but strongly expressed the E2F1 factor. Interestingly, we obtained an E2F1 overexpression in breast cancer tissues, and in the same way, the average H19 expression tended to be up-regulated in neoplasms. Thus,

Fig. 7. H19 expression promotes cell proliferation. A, H19 overexpression in MDA-MB-231 clones. Total RNA of a mock-transfected (Neo) and the two H19-transfected clones (S14-3 and S14-4) were isolated and reverse-transcribed. Real time PCR amplifications were performed using a SYBR Green mix. The curves represent fluorescence versus cycle number of the target H19 gene and the reference RPLP0 gene in Neo, S14-3, and S14-4 cDNAs. Cycle threshold determination was carried out at fluorescence level of 100. B, normalization of H19 expression. Relative H19 expression was calculated as indicated under "Materials and Methods" using the RPLP0 gene as reference. C, H19 promotes S-phase entry in MDA-MB-231 cells released from quiescence. MDA-MB-231 cells stably transfected with H19 were serum-starved for 48 h and subsequently stimulated with fresh medium containing 10% FCS. At the indicated times, the cells were harvested and assayed for cell cycle distribution by FACS. Neo (black bars) corresponds to a mock-transfected cell line, and both S14-3 (gray bars) and S14-4 (white bars) correspond to H19-transfected cell lines. D, H19 accelerates MDA-MB-231 cell growth. Cells were plated (T0), serum-starved for 24 h, and grown in 10% FCS medium for 48 h (T48). All values shown represent the mean values of three independent experiments.
it appears that gene expressions do not result in a single concomitant regulation. It is highly likely that other factors intervene in this complex regulation network. Consequently, we chose these cells to stably overexpress the H19 gene and to further investigate its function in cell growth.

Indeed, H19 overexpression in the MDA-MB-231 cell line accelerates the cell cycle progression, by increasing S-phase entry in asynchronously growing cells as well as in cells entering the cell cycle from quiescence. The inhibition of H19 expression by specific siRNA in these MDA clones suggests the direct contribution of the H19 gene to this cellular process and its involvement in a rate-limiting step in the G1/S-phase transition. Furthermore, knock-down of the endogenous H19 RNA in MCF-7 cells has a significant impact on cell proliferation. These data indicate that H19 is involved in cell cycle progression in physiological conditions. We can at least partly explain this function by our previous finding that thioredoxin is upregulated by H19 in MDA-MB-231 cells (13). Numerous studies argue for a role of thioredoxin in cell proliferation and transformation. This protein possesses a growth factor activity and has been reported to be overexpressed in a number of human primary cancers (64, 65). Furthermore, thioredoxin transfected in breast cancer MCF-7 cells increased colony formation in soft agar and tumorigenesis in immunodeficient mice (66). The protein, added to minimal culture medium, stimulates the proliferation rate of these cells (67). Interestingly and in line with

**FIG. 8.** H19 knock-down by RNA interference reduces cell proliferation. A, effect of siRNA sequences on H19 expression. S14-4 cells were transfected with 400 pmol of siH19A or siH19B alone or in combination for 48 h. Transfection of the siGFP sequence was used as a control condition. Total RNA were isolated and processed for real-time PCR. B, normalization of H19 expression. Relative H19 expression was calculated as indicated under “Materials and Methods” using the RPLP0 gene as reference. C, H19 inhibition reverse the growth phenotype of the H19-transfected cells. S14-4 cells were plated on day 1 (D1), transfected with siGFP (S14–4siGFP) or the siRNA mix (S14–4siH19) on day 2 (D2), and cultivated in 5% FCS medium until day 5 (D3–D5). In parallel, nontransfected S14-4 and Neo clones were used as positive controls. Statistical significance is indicated by asterisks as described under “Materials and Methods” and calculated for each day for S14–4siH19 with regard to the corresponding control condition S14–4siGFP. D, H19 promotes S-phase entry. S14-4 cells were transfected with the control siGFP or with the mix of siH19, serum-starved for 24 h, and submitted to a FACS analysis. The cell distribution in the different phases of the cell cycle is indicated as a percentage of total cells. E, RNAi on endogenous H19 in MCF-7 cells reduces cell proliferation. MCF-7 cells were plated on day 1 (D1), transfected with siGFP or the siRNA mix on day 2, and cultivated in 5% FCS medium until day 5 (D5), when cells were counted. Statistical significance is indicated by asterisks as described under “Materials and Methods” and calculated for D5 for siH19 with regard to the corresponding control condition siGFP.
this result, Ayesh et al. (25) have shown a growth advantage for bladder carcinoma cells overexpressing H19 and cultured in serum-poor medium. This advantage was due in part to the inability of the H19-overexpressing cells to induce the cyclin-dependent kinase inhibitor p57kip2. The authors also revealed an up-regulation of cell cycle regulator genes, including genes involved in DNA synthesis, by H19. Therefore, considering all of these data and in agreement with the results presented here, H19 exerts its growth-promoting effect through its ability to modify the level of cell cycle molecules, and thioredoxin could be one of the mediators in this effect.

In conclusion, our present findings demonstrate that H19 promotes the G1-S transition in breast cancer cells through a link to the E2F1 factor. Fig. 9 is a comprehensive overview of our results. This work is the first documented report of the contribution of an mRNA-like noncoding RNA in cell cycle progression. Indeed, H19 can promote S-phase entry in malignant cells under various conditions, and these observations provide the evidence that H19 expression is an active partner and not simply a consequence of cell proliferation. H19 overexpression confers an obvious advantage for cell proliferation and hence reinforces the aggressive phenotype of neoplastic cells. These properties explain the oncogenic function of H19 in breast cancer genesis.

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Fig. 9. Schematic representation of the various intermediates which take part in H19 cell cycle promotion. The dotted arrows represent previously established data confirmed in our model. The filled arrows represent data established in this work.
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