The expression of the H19 gene and its function in human bladder carcinoma cell lines

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Abstract The human H19 gene is a paternally imprinted oncofetal gene, highly expressed in several fetal tissues, down-regulated in nearly all adult tissues but re-expressed in carcinomas of tissues which express the gene in fetal life. It has no known protein product and till today, no function could be designated to H19 RNA. Cells derived from bladder carcinomas and hepatocellular carcinomas were transfected with plasmids carrying a luciferase reporter gene under the control of a 800 nucleotides long promoter region of the H19 gene either alone or together with different parts of a 5 kb downstream region, previously shown to possess enhancer activity. Our results provide evidence that three regions of the 3′ downstream sequence can independently stimulate the H19 promoter activity in a tissue and cell specific manner. The growth rate of two cell populations, both derived from the same bladder carcinoma cell line and which differ in their H19 RNA content, were compared. The cells with a high H19 RNA level stopped their proliferation after 48 h when cultivated in a low serum containing media while the cells lacking H19 RNA continued their proliferation for at least an additional 48 h period.

Key words: H19 gene; H19 enhancer; H19 function; Bladder carcinoma

1. Introduction

The human H19 gene is located on chromosome 11p15.5. It is one of a cluster of at least seven imprinted genes, six of them are expressed from the maternally inherited allele, the only exception is the paternally expressed IGF-2 gene [1].

The H19 gene encodes a polyadenylated transcript which is transported to the cytoplasm. Recently, it was reported that H19 RNA is associated with polyribosomes in different human cells [2]. However, no protein product of the H19 gene has been detected till now and the 256 amino acid long protein, the potential product of the open reading frame of the human H19 gene, does not share any amino acid sequence homology with the potential protein products of the mouse and rat H19 open reading frames [3]. Notwithstanding its very high abundance in some tissues, we are still ignorant about its functions.

Upon the basis of several experimental findings, a role as tumor suppressor has been proposed for the H19 gene [4–6]. However, recently, we and others have reported findings that are contradictory to such a proposal. The H19 gene was found to behave as an oncofetal gene, a gene highly expressed in embryonic tissues, not or very low expressed in tissues of the normal adult, but expressed in tumors derived from tissues which expressed the gene during embryonic development [7–9]. We injected cells from human bladder carcinoma and choriocarcinoma cell lines, which did not express H19, into nude mice. In the tumors which developed, H19 was expressed at an easily detectable level [10–12]. The significant increase in H19 expression during the process of tumor formation in the mice raises the question if the H19 gene product fulfills a role in the process of tumorigenesis.

In order to answer this question, we stably transfected cells from a bladder carcinoma cell line, which did not express H19, either with an episomal construct in which H19 expression is under the control of the CMV promoter, known to be highly active in cell lines derived from human bladder carcinoma, or with a similar construct not expressing the H19 gene.

In the experiments described, we showed a very significant difference in the response of the cells to a low serum content of the growth medium. The H19 RNA containing cells stopped their proliferation after 48 h in contrast to the cells lacking H19 RNA.

In a recent work, we reported that in cells of the choriocarcinoma-derived cell lines JAr and JEG-3, the transcription of the human H19 gene is under the control of a 5 kb long 3′ downstream enhancer region [13]. Similar results were previously reported for the mouse gene by Yoo-Warren et al. [14]. We have extended our investigation on this 5 kb enhancer region and have sequenced the whole 5 kb region mentioned above and studied its action on the H19 promoter in human bladder carcinoma cell lines. In order to study the sequences responsible for the enhancer effect in greater detail, we report here about the enhancer activity of different parts of the above mentioned 5 kb region. These experiments were carried out using the highly sensitive luciferase reporter gene assays. The results obtained provide evidence that the human H19 5 kb 3′ downstream enhancer region contains three sequence motifs which can independently stimulate transcription from the H19 promoter.

2. Materials and methods

Human bladder carcinoma cell lines HT-1376, UM-UC-3, T24P, 5637, RT-112 and hepatocellular carcinoma cell lines Huh7 and Hep3B were obtained from the American Type Culture Collection,

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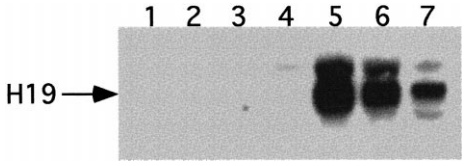

Fig. 1. Expression of the H19 gene in T24P and transfected T24P cells. Autoradiogram of Northern blots containing RNA isolated from T24P cells (lane 1), T24P cells containing the non-H19 expressing episomal vector (TA11, TA12 and TA13, lanes 2–4) and T24P cells containing a H19 expressing episomal vector (TA31, TA32 and TA33, lanes 5–7). A second autoradiogram was produced, using sense H19 RNA as probe. No antisense RNA was detected in any of the cell populations (results not shown).

USA. The EJ28 cell line was obtained from the German Cancer Research Center (Heidelberg, Germany). The cells were grown as previously described [13].

For measurements of the cell culture growth rate, the cells were seeded at an initial density of 0.2×10^6 cells into dishes, harvested after designated periods of time and counted. Cells were grown at different FCS concentrations, as indicated in legends.

Preparation of probes for detection of sense and antisense H19 RNA and Northern blotting was carried out as described in [13].

2.1. Luciferase reporter gene constructs

All the luciferase gene reporter constructs were built from the pGL3 basic (Luc-1) vector (Promega) which lacks both eukaryotic promoter and enhancer sequences. The H19 promoter cloned in the *Eco*RV site of the pBluescript II SK (designed pbh 19 p#1) was obtained from Dr Ekkehard Schulze. The H19 promoter region from nucleotide −819 to +14 was amplified by PCR from the pbh 19 p#1, using primers 5′-ATATGGTACCGACAACCCTCACCAAAG-3′ (upstream) and 5′-ATATAAGCTTCTTCTCCCTCACCCTGCTC-3′ (downstream).

In order to facilitate subsequent cloning of the PCR product, *Kpn*I and *Hin*dIII enzyme restriction sites were incorporated into the PCR primers design. The resulting PCR product was digested with the *Kpn*I and *Hin*dIII enzymes and ligated into the *Kpn*I and *Hin*dIII sites of the Luc-1 basic vector, yielding the Luc-PBH19 construct.

Reporter gene plasmids containing different H19 enhancer regions downstream of the H19 promoter-Luc reporter gene were constructed as follows. The plasmid PH19EH19D (described in [13]) containing the 5 kb H19 downstream enhancer was digested with *Bam*HI yielding two fragments, a 4.1 kb fragment at the 5′ end and an 0.9 kb fragment at the 3′ end of the 5 kb region.

The Luc-PBH19-4EH19 and the LucPBH19-0.9EH19 constructs were prepared by insertion of the 4.1 kb and the 0.9 kb *Bam*HI fragments, respectively, into the *Bam*HI site of Luc-PBH19 plasmid, downstream to the H19 promoter/Luc reporter gene. The sequence of the 4.1 kb fragment shows the presence of two BSTXI restriction sites (at nucleotides 1206 and 3334), the construct Luc-PBH19-4EH19 was digested with BSTXI deleting a fragment between nucleotides 1206 and 3334 of the 4.1 kb region. Following self-ligation of the plasmid, the expression vector Luc-PBH19-2EH19, which contains a 2 kb H19 fragment, was formed.

The plasmid PH19EH19D was digested with *Sac*I, releasing the 5.0 kb H19 enhancer fragment. This enhancer and the linearized plasmid Luc-1 were both converted to a blunt end terminus by T4 DNA polymerase, followed by ligation yielding the Luc-PBH19-5EH19 construct.

2.2. H19 expression construct

A H19 expression vector was constructed as follows: a 6 kb fragment of the human H19 [6] was cloned into the multiple cloning sites of the pBKC-BKV (Genetech) episomal expression vector under the control of the CMV promoter. The 6 kb H19 fragment spans the complete transcribed region of the human H19 gene and begins 48 nucleotides upstream of the transcriptional start site. An 800 nucleotides fragment derived from the 3′ portion of the transcribed region of the human H19 gene [13] was inserted in the pBKC-BKV vector in an antisense orientation, providing us with a pBKC-BKV non-H19 expression control construct (see Fig. 1). The pBKC-BKV vector contains the gene for G418 resistance.

Table 1
Stimulatory effect of different H19 3′ downstream regions on the H19 promoter activity in bladder carcinoma-derived cells

Cell line	H19 3′ downstream regions in the luciferase reporter gene construct			
	0.9 kb	4.1 kb	5.0 kb	2.0 kb
T24P	1.5	9.0	5.3	4.0
RT112	1.0	12	7.0	2.5
5637	11	4.4	15	2.0
EJ28	1.7	6.0	4.0	2.5
HT1376	17	20	32	20
UM-UC-3	9.0	7.0	10	1.1

In all luciferase gene constructs, a H19 promoter region preceded the luciferase gene (see Section 2). The activity of the different H19 3′ downstream regions in each cell line are calculated on the basis of activity = 1.0 for luciferase activity of the construct in which the reporter gene was under control of the H19 promoter alone.

2.3. Transfections

Transient transfections were carried out as previously described [13].

In the case of stable transfections of the T24P cells, 24 h after the transfections with the pBKC-BKV episomal vectors, 0.5 mg/ml G418 (Geneticin) was added to 1.0 ml of the culture to initiate selection.

2.4. Luciferase assay

Cells carrying the reporter gene construct were harvested after 48 h (20–100 μg) and equal amounts of protein were examined for luciferase activity using the Promega kit, 'Luciferase Assay System' (E1500-PROMEGA, USA). The light output was detected by the Lumac Biocounter apparatus.

2.5. Sequencing

Sequencing was carried out on both strands of the DNA, using the Automated DNA sequencing, Ye terminator cycle sequencing procedure (ABT Prism 379, Perkin Elmer) DNA sequencer.

3. Results

We sequenced a 5.0 kb 3′ downstream region of the human H19 gene which was kindly donated to us by Dr S.M. Tilghman (Princeton University, USA). The sequence of a 4.1 kb long fragment starting at the 5′ end of this region was submitted to GenBank (accession number AF091107). Recently, the sequence of a 41 kb long region of human chromosome 11p15.5 was reported by Ishihara et al., GenBank accession number AF087017 [15]. This sequence covered the H19 gene region from nucleotide −8325 till +29 571. The sequence of the 5.0 kb fragment that we determined was nearly 100% identical to the corresponding sequence reported by Ishihara et al. [15]. These data enabled us to accurately locate the 5.0 kb enhancer region used in our experiments, relatively to the transcribed region of the H19 gene (from nucleotide 6033 till +10 972).

In a previous communication [13], we reported that in cells derived from human choriocarcinomas, the activity of the

Table 2
Stimulatory effect of different H19 3′ downstream regions on H19 promoter activity in hepatocellular carcinoma-derived cell lines

Cell line	H19 3′ downstream region in the luciferase reporter gene construct			
	0.9 kb	4.1 kb	5.0 kb	2.0 kb
Huh7	1.0	13	13	4.0
Hep3B	1.0	45	38	8.0

Details as in Table 1.

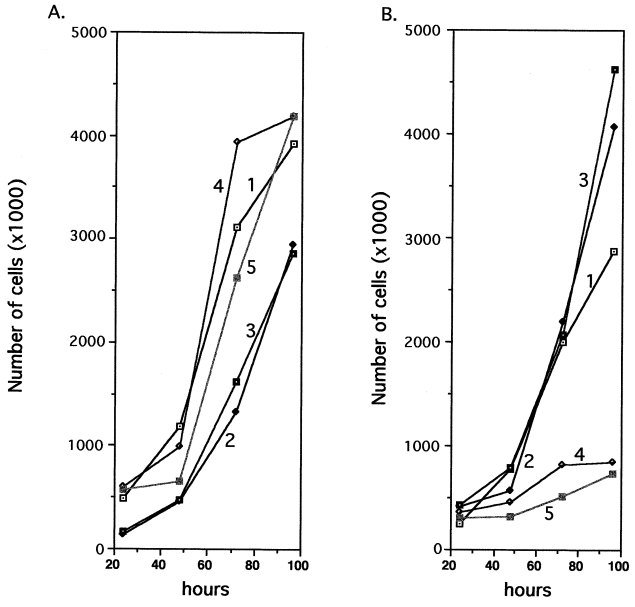

Fig. 2. Growth rates of T24P cells and T24P cells transfected with a H19 expression episomal construct (TA31 and TA32) or with a control episomal construct (TA11 and TA12). (A) Cells grown in medium containing 10% FCS. (B) Cells grown in medium containing 0.1% FCS. (1) T24P untransfected cells, (2) TA11 cells, (3) TA12 cells, (4) TA31 cells, (5) TA32 cells

H19 promoter is stimulated by (a) sequence(s) in a 5.0 kb H19 3′ downstream region. This region is located from nucleotide +6033 till +10 972 relative to the H19 transcriptional start sign. Moreover, we showed that this region also mediates the induction of H19 gene expression in cell lines derived from human testicular germ cell tumors by retinoic acid [13].

The results summarized in Table 1 show that the same 3′ downstream region can activate the H19 promoter in cells of six bladder carcinoma cell lines. The 5.0 kb region was cut into two non-overlapping fragments, one of 0.9 kb and one of 4.1 kb. The 4.1 kb fragment, when incorporated into a luciferase reporter gene construct in which transcription of the luciferase gene is under the control of the H19 promoter, stimulated luciferase gene transcription in cells of all the six cell lines tested (Table 1). The 0.9 kb fragment strongly enhances H19 promoter activity in three of the six cell lines, but had only a small stimulatory effect in two cell lines and no activity at all in one of the cell lines (Table 1). In cell lines in which the 0.9 kb fragment has a large enhancing effect on activity of the H19 promoter, the effect of the 4.1 kb fragment was significantly smaller than that of the 5.0 kb fragment. Opposite results were obtained in those cells in which the effect of the 0.9 kb fragment was marginal or absent. The 2.0 kb fragment increased the activity of the H19 promoter in cells of five cell lines but its effect was smaller than that of the 4.1 kb region. In one cell line, we did not observe any stimulatory effect.

We repeated the experiments with cells from two cell lines derived from human hepatocellular carcinoma (Table 2). We found no activity of the 0.9 kb region, the 4.1 and 5.0 kb regions had the same or nearly the same stimulatory effect on the H19 promoter activity and the 2.0 kb region had a smaller effect than both the 4.1 and 5.0 kb regions.

In order to gain more information about the function of the H19 gene product in the cell, we stably transfected cells of the T24P bladder carcinoma cell line with either an H19 expression construct or a similar construct not expressing the H19 gene (see Section 2). Fig. 1 shows the level of H19 RNA in those cells and in untransfected T24P cells.

We measured the growth rate of the above mentioned cells. The cells were cultivated in two different media, one containing 10% FCS and the other 0.1% FCS. At the high FCS concentration, transfected cells and the T24P parent cells grew at approximately the same rate during a 4 day culture period (Fig. 2). However, the cells transfected with the H19 expression construct grew much slower in 0.1% FCS containing medium than the cells containing non-H19 expression constructs or the parent T24P cells. Actually, their proliferation was nearly completely inhibited after 48 h of culture (Fig. 2). We wish to emphasize here that the same results as shown in Fig. 2 were obtained in six independent experiments, starting from establishment of the stably transfected T24P cell lines. The H19 RNA level in cells expressing H19 from the expressing vector and grown in a 10% FCS containing medium was equal to that of cells grown in 0.1% FCS containing medium (Fig. 3).

Similar results were obtained using NCCIT cells, a cell line derived from a Testicular Germ Cell Tumor, transfected with the same episomal constructs as used in the above described experiments. In these cells, H19 RNA could be detected only in NCCIT cells transfected with the H19 expressing construct (results not shown).

4. Discussion

All our observations summarized in Tables 1 and 2 indicate the presence of at least three different enhancer elements in the 5 kb 3′ downstream region of the human H19 gene.

However, the capacity of these enhancer elements to activate the H19 promoter seems not only to be tissue specific but also to differ among cell lines derived from carcinomas of the same tissue.

One of those enhancer elements, the one located in the middle of the 4.1 kb fragments, was found to be active in all cell lines tested. A second enhancer element, contained in the 0.9 kb region, was not active in the two cell lines derived from hepatocellular carcinomas and in one of the bladder carcinoma-derived cell lines.

A third enhancer element located in the 3′ region of the 4.1 kb fragment (and included in the 2.0 kb fragment) was active in all (except one) of the cell lines tested.

Yoo-Warren et al. [14] found in the mouse H19 3′ downstream region at least two separate enhancer elements. One of them is contained in a 269 nucleotides fragment located be-

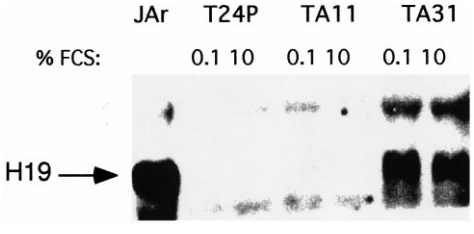

Fig. 3. Expression of the H19 gene in T24P and transfected T24P cells grown at different FCS concentrations. Cells were grown for 4 days in 0.1 or 10% FCS and H19 expression was examined in Northern blots. For TA11 and TA31, see legend Fig. 2.

tween +8 and +9 kb, relative to the H19 gene transcriptional start site, the second is 700 nucleotides further downstream and is contained in a 297 nucleotides fragment. We found two regions in the 4.1 kb fragment of the human H19 gene used in our experiments which bear a high percentage of sequence identity with these two mouse enhancer regions. The first is a 189 nucleotides fragment (between nucleotides +7658 and +7847) with a 70% sequence identity with one of the mouse enhancers. A second region is located between nucleotides +9375 and 9674 with a sequence 78% identical to the second mouse enhancer region. This fragment is also contained in our 2.0 kb fragment. This conservation of synteny supports our assumption about the presence of two enhancer sequences in the 4.1 kb region, one located in the middle, the second in the 3′ end of this region.

In our present investigation, we have compared the growth rate of two cell populations, both derived from the T24P bladder carcinoma cell line, one expressing the H19 gene and the second containing neither sense nor antisense H19 RNA. We observed a very significant difference between growth rates of cells containing H19 RNA and those of cells lacking H19 RNA (Fig. 2). We assume that H19 RNA prevents the cell from continuing proliferation under unfavorable conditions, namely the lack of one or more growth factor(s) obligatory for normal proliferation. It helps cells to avoid growth under unfavorable conditions, the lack of serum factors, which will ultimately lead to cell death. Probably, H19 RNA can promote expression of genes which limit the growth rate under certain stress conditions such as lack of one or more serum factors. It is known that expansion of primary tumors and metastases is critically dependent upon an adequate supply of serum factors. As a result, both are highly dependent upon neovascularization [16]. If our assumption is true, then, H19 RNA itself plays an important role in the regulation of tumor cell growth under physiological conditions, namely temporary lack of an adequate supply of serum factors.

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