The effect of retinoic acid on the activation of the human H19 promoter by a 3′ downstream region

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Abstract The human H19 is paternally imprinted (maternally expressed). It is transcribed by RNA pol II, but has no protein product. Its function is unknown. We showed that the transcription of the human H19 gene is under the simultaneous control of both a 5′ upstream (promoter) region and a 3′ downstream region in cell lines derived from human choriocarcinomas. Moreover, the activation of the H19 promoter by retinoic acid in cells derived from human testicular germ cell tumors is dependent upon the 3′ downstream region. The possibility that the action of retinoic acid on the H19 promoter is an indirect one and involves a member of the AP2 transcription factor family is discussed.

Key words: Interaction of 3′ and 5′ regions of human H19 gene; Testicular tumor stem cell; Retinoic acid; Choriocarcinoma cell line

1. Introduction

The H19 gene was first described as a gene expressed in the mouse liver which shared a common control mechanism with the α-fetoprotein gene [1]. The human H19 is paternally imprinted (maternally expressed) and located on chromosome 11p15.5. This region is now known to include at least six additional imprinted genes. In the human the H19 gene is highly expressed in several embryonic and fetal tissues, including the fetal adrenal, liver, and in the placenta [2,3]. Expression is nearly completely down-regulated postnatally but it is interesting to note that H19 is expressed in the adult in the endometrium during the luteal phase of the menstrual cycle [4].

The H19 gene is transcribed by RNA pol II, its primary transcription product undergoes processing and is transported to the cytoplasm. The H19 gene very likely does not code for a protein [5]. Both human and mouse H19 RNAs have open reading frames (ORFs) which can potentially code for 256 and 132 amino acid long peptides, respectively. However, there does not exist any similarity between the two amino acid sequences which can be predicted. Nothing definite is known about the function of the H19 RNA. A tumor suppressor role was proposed for the H19 gene [6] but in our studies and that of others evidence has been provided which contradicts this proposal. The H19 gene is expressed in a wide variety of carcinomas, such as bladder carcinoma [7], lung cancer [8], hepatocellular carcinoma [9] and testicular germ cell tumors [10]. Moreover, upon injection of cells from clones derived from choriocarcinoma cell lines (JAr and JEG-3) into nude mice, tumors were formed which expressed enhanced levels of H19, including cases in which the cells injected did not express detectable H19 mRNA when grown in culture [11]. Similar results were obtained upon injection of cells of different human bladder carcinoma derived cell lines into nude mice [12]. All these observations led us to the conclusion that the H19 gene is not a tumor suppressor gene as has been previously claimed [10,13].

Little is known about the regulation of the human H19 transcription. The H19 promoter activity in the mouse is under the control of 5′ upstream regulatory elements and 3′ downstream region(s) located between 5 and 6.5 kb 3′ of the polyadenylation site [14]. We found that cAMP and agents which induce cAMP formation and activate protein kinase A (ACTH, cholera toxin, forskolin) increase the H19 RNA level, while a protein kinase C activating agent, such as TPA, decreases the H19 RNA level in primary human fetal adrenal cells [15].

It is known that the sequences of the human and mouse H19 5′ upstream regions, which carry paternal specific methylation patterns, differ widely [16,17].

In this work all our experiments deal with either the contact human H19 gene or with 5′ upstream and 3′ downstream regions derived from the human gene, using cell lines of human origin. This work is part of a program to study the interaction between 5′ upstream and 3′ downstream regions of the human H19 gene which govern the regulation of the human H19 promoter activity. This interaction will very probably differ from that in the mouse because of the above mentioned species specifity of the 5′ upstream regulatory regions of the H19 gene.

Retinoic acid plays an important role in the establishment of the differentiation pattern during embryogenesis. In cells retinoic acid binds to one (or more) members of the retinoic acid receptor family. Some of the genes regulated by these receptors code for transcription factors. Therefore retinoic acid can affect the transcription level of genes by a direct or indirect mechanism [18]. NT2 cells (derived from a human testicular germ cell tumor) do not express the H19 gene. Retinoic acid induces H19 expression in these cells [19]. Therefore, it is possible that H19 RNA mediates, at least partly, the effect of retinoic acid on the differentiation of various tissues during embryogenesis, especially in the light of our previous finding that the H19 expression level is positively correlated with the differentiation stage of placental cytotrophoblasts both in vivo and in vitro [20]. One of the aims of the present studies is to investigate the regulation of the human H19 gene

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transcription by 5′ upstream and 3′ downstream regions in the presence of retinoic acid.

2. Materials and methods

2.1. Cell culture

The NT-2 and NCCIT cell lines were maintained in DMEM-F12 (1:1) medium containing 10% fetal calf serum (inactivated, 55°C for 30 min), 25 mM HEPES (pH 7.4), penicillin (180 units/ml), streptomycin (100 μg/ml) and amphotericin B (0.2 μg/ml). 4×10^4 cells/cm^2 were plated in polystyrene culture dishes (NUNC). Every 4 days the cells were trypsinized with 0.05% trypsin-EDTA solution (Beit Haemek) for 15 min and replated again at the same initial density. 2×10^5 cells were seeded in 30-mm wells and grown for four days. Every 24 h the cells from two wells were harvested as described above and counted.

2.2. Preparation of the probe for H19 RNA detection

A region of 800 bp upstream from the poly(A) site of the human H19 gene was subcloned into the polylinker sites of *Eco*RI and *Hin*dIII of the Bluescript II SK$^+$ plasmid (Stratagene) between the T_7 and T_3 RNA polymerase binding sites. The plasmid was linearized by digestion with *Hin*dIII. Antisense and sense RNA transcripts labeled with DIG nucleotides were produced in vitro, using the Amersham Kit, T_7 and T_3 RNA polymerases from Boehringer (Mannheim) according to the manufacturer's instructions. The fragments were separated from unincorporated nucleotides by ethanolic precipitation.

2.3. Northern blotting

Total cellular RNA was isolated from cells according to [21]. Five or 10 μg of each RNA sample was separated by 1% agarose-formaldehyde gel electrophoresis and transferred to Hybond-N Nylon filters (Amersham, England). The blots were stained with methylene blue, in order to ascertain that equal amounts of RNA had been loaded in each lane. The blots were prehybridized at 60°C in 50% formamide, 5× SSPE, 5× Denhardt solution, 0.1% SDS with 0.1 mg/ml herring testes DNA followed by hybridization with specific probes for 16 h. The detection was done by the alkaline phosphatase method from the DIG Nucleic Acid detection kit (Boehringer). Sense H19 RNA was used for control and failed to produce any signal.

2.4. Plasmids

The control plasmids pCAT-basic and pCAT-enhancer vectors were purchased from Promega. Control plasmid pCAT-basic (Cat. No. E 1041) lacks eukaryotic promoter and enhancer sequences and the pCAT-enhancer vector (Cat. No. E 1021) contains an SV40 enhancer element built into the plasmid backbone. The H19 promoter plasmids were prepared as follows. A region of 814 bp immediately upstream of the human H19 transcriptional start site was amplified by polymerase chain reaction (PCR) as described in [22] and cloned into the commercial reporter-gene vectors (Promega). A genomic *Sac*I fragment (5-kb length, mentioned as the 3′ downstream region), which contained a 300-bp sequence with homology at least to one of the mouse H19 3′ enhancer regions, was donated by S. Tilghman, Princeton, USA and ligated 3′ to the CAT gene.

2.5. Transfections

A total of 0.3×10^6 cells in 30-mm dishes were transfected with 7 μg of plasmid using the calcium phosphate precipitation method [23]. The precipitates were added to the cells with 1 ml of the usual growth medium. After 16 h of incubation, the medium was changed to fresh normal medium, with or without all-*trans* or 9-*cis* retinoic acid. Control cells received normal medium with 0.1% ethanol.

2.6. CAT assay

After 24–96 h of culture, cells were harvested and lysed. Aliquots containing equal amounts of protein were analyzed for CAT activity, using butyryl CoA as substrate, according to the organic phase extraction procedure [24].

3. Results

The expression of the H19 gene in the mice is under the control of both 5′ upstream and 3′ downstream sequence elements. It was shown that for maximal expression both the promoter region and the 3′ enhancer regions were obligatory in mouse cells from endodermal and mesodermal origin [14]. We prepared CAT constructs in which CAT transcription was under the control of the human H19 gene 5′ upstream (−814 to +5) and/or a 5-kb downstream region (+6 to +11 kb) as presented in Fig. 1.

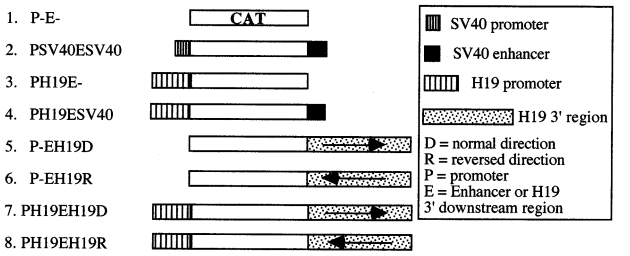

Fig. 1. A schematic presentation of the insertions introduced in the CAT reporter plasmid (Promega, USA).

The choriocarcinoma cell lines JAr and JEG-3 were transiently transfected with these constructs. The cells were incubated for 48 h and CAT activity measured (Fig. 2). The 5′ upstream region alone caused a small increase in the CAT activity, but no activity of the CAT enzyme could be detected in cells in which the CAT gene was under the control of the H19 3′ downstream region alone. However, a considerable increase in the CAT activity was observed when both the H19 promoter region and the 3′ downstream region were included in the CAT expression construct. In JEG-3 cells there was a 6.0–6.5-fold increase in CAT activity and in JAr-cells a

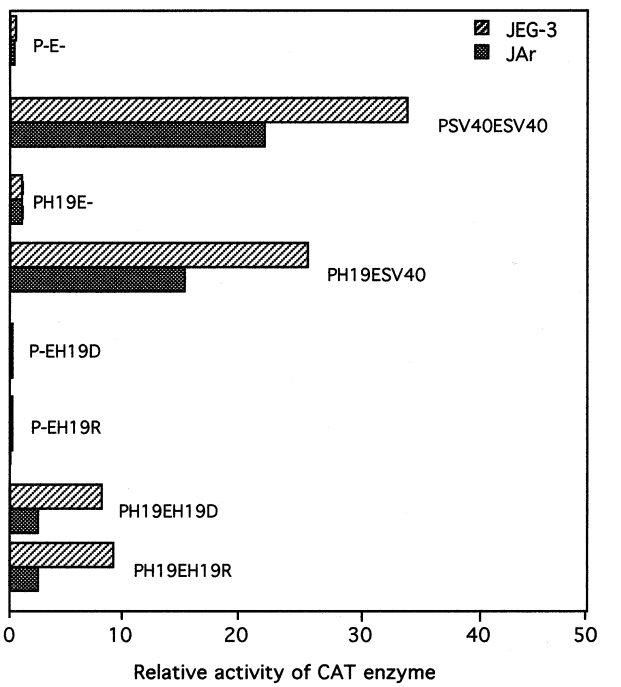

Fig. 2. Enhancement of the CAT reporter gene activity by H19 5′ and 3′ downstream sequences in JAr and JEG-3 cells. JAr and JEG-3 cells were transiently transfected with the plasmids presented in Fig. 1. The cells were collected after 48 h and CAT activity was analyzed. The CAT activity obtained by the H19 5′ region (promoter) alone is designated as 1. Nearly equal results were obtained from four independent experiments.

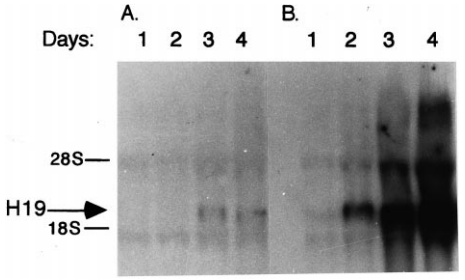

Fig. 3. Induction of H19 expression by all-*trans* retinoic acid in NT-2 cells as a function of incubation time. Shown are Northern blots of NT-2 RNA derived from cells cultivated in the absence (A) or in the presence of 10 μM all-*trans* retinoic acid (B). The numbers above the lanes indicate incubation time (days).

2.0–2.5-fold increase due to the presence of the 3′ downstream region. The 3′ region exhibited its stimulatory effect when inserted in the plasmid both in a direct and reversed orientation. The SV40 enhancer showed a higher stimulatory activity than the H19 3′ downstream region sequence in both the JEG-3 and JAr cells. These observations show unequivocally that the human H19 gene transcription similar to that of the mouse H19 gene is under the control of 3′ downstream regions which cooperate with H19 promoter 5′ upstream regions.

We also investigated the effect of retinoic acid on the activation of the H19 promoter by an H19 3′ downstream region, in JAr cells transfected with the appropriate CAT constructs. However, the addition of retinoic acid to the medium of the transfected cells did not cause any increase in the CAT activity (data not shown). We showed before that retinoic acid stimulates H19 expression in NT-2 cells which are embryonic stem cells derived from a human testicular tumor which do not express the H19 gene in the absence of retinoic acid [19]. All-*trans* retinoic acid induces the expression of H19 in these cells as can be seen from Fig. 3. Maximal increase was observed after the cells were incubated for 96 h in the presence of retinoic acid. During the 96 h of incubation the number of cells increased between 4- and 6-fold, both in the absence and in the presence of retinoic acid. This was determined by counting the number of the cells in each well as a function of incubation time (results not shown). In the NCCIT cell line a similar phenomenon was observed, however, the kinetics of the induction of H19 expression was considerably slower. H19 expression was still negligible after 72 h of incubation and was

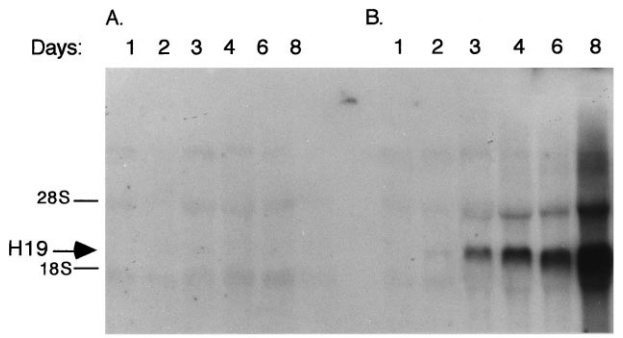

Fig. 4. Induction of H19 expression by all-*trans* retinoic acid in NCCIT cells as a function of incubation time. Shown are Northern blots of NCCIT RNA derived from cells cultivated in the absence (A) or in the presence of 10 μM all-*trans* retinoic acid (B). The numbers above the lanes indicate incubation time (days).

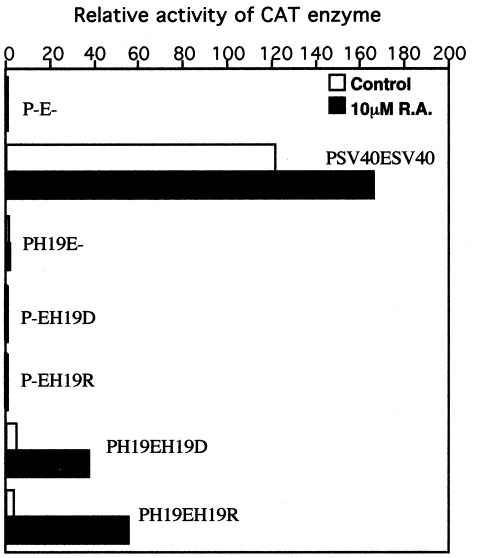

Fig. 5. Activation of CAT expression by H19 5′ upstream promoter and 3′ downstream sequences in NT-2 cells in the absence or presence of all-*trans* retinoic acid. The cells were transiently transfected with plasmids presented in Fig. 1. The cells were collected after 72 h and CAT activity was analyzed. The CAT activity obtained by the H19 promoter alone is designated as 1. Identical results were obtained in two independent experiments.

low after 96 h, but continued to increase for at least up to 8 days (Fig. 4). It is interesting to note that in the cells of the two cell lines, two additional bands hybridizing with our H19 antisense probe appeared in addition to the 'normal' 2.3-kb H19 band. Retinoic acid induces the three above mentioned transcripts with similar kinetics. However, in JAr cells we have also encountered three bands in the absence of retinoic acid treatment [11]. We do not know the nature of these RNAs. They do not hybridize with an antisense probe directed against the sense RNAs derived from the 814-bp long H19 5′ upstream region.

In order to gain more information about the mode of the retinoic acid stimulatory action on the human H19 promoter activity, NT-2 and NCCIT cells were transiently transfected with the different constructs described in Fig. 1. In the two transfected cell lines the activity obtained from the reporter gene showed a low but consistent increase when both the 5′ H19 upstream and 3′ downstream regions were included in the CAT reporter gene construct. As can be seen from Figs. 5 and 6, both the H19 5′ upstream region and the H19 3′ downstream region alone are not sufficient to promote H19 expression either in the absence or in the presence of retinoic acid in both cell lines. The addition of retinoic acid to those cells, however, caused a very significant additional increase in the CAT enzyme activity obtained from constructs containing both the 5′ upstream and the 3′ downstream regions. Retinoic acid had no effect at all on the CAT activity in cells with constructs from which the 3′ downstream region was omitted (Figs. 5 and 6). Yoo-Warren et al. [14] reported similar results using 5′ upstream and 3′ downstream regions of the mouse H19 genes in the PC13 mouse embryonal carcinoma cell line. Two different 3′ region constructs were used in our experiments, one with the 3′ region sequence in the direct, the second one in the reverse direction. CAT constructs with the 3′ region in the reverse direction had a higher activity than the

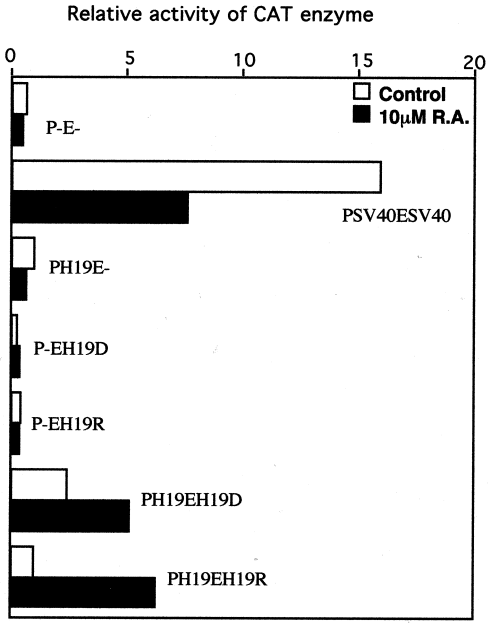

Fig. 6. Activaton of CAT expression by H19 promoter and 3′ downstream sequences in NCCIT cells in the absence or presence of all-*trans* retinoic acid. The cells were transiently transfected with plasmids presented in Fig. 1. The cells were collected after 96 h and CAT activity was analyzed. The CAT activity obtained by the H19 promoter alone is designated as 1. Identical results were obtained in two independent experiments.

CAT constructs containing the 3′ region in the direct orientation (Figs. 5 and 6) in six independent experiments. The inducing effect of retinoic acid on CAT activity in the NT-2 cells was higher than that in the NCCIT cells (10-fold and 5-fold, respectively, Figs. 5 and 6). In one exceptional case we obtained a negative retinoic acid effect with a CAT construct under the control of both SV40 promoter and SV40 enhancer in NCCIT cells (Fig. 6). Since the transfection was a transient one, we could not extend the experiments for more than 4 days. The difference in the level of the induced CAT activity in the two cell lines is very likely related to the observed difference in the kinetics of endogenous H19 RNA accumulation as shown in Figs. 3 and 4, which clearly show that the maximal H19 RNA level appears much earlier in NT-2 cells than in NCCIT cells. We routinely used retinoic acid at a 10^{-5} M concentration but the retinoic acid effects were not smaller at 10^{-6} M. Moreover, 9-*cis* retinoic acid was as active as all-*trans* retinoic acid in the promotion of the reporter gene activity (results not shown).

4. Discussion

As previously reported by us, we isolated different clones from JEG-3 cell lines that showed large differences in their H19 RNA levels [11]. When two of these clones, which differ greatly in their H19 RNA levels, were transfected with the constructs mentioned above, a 6-fold increase in CAT activity was induced by the 3′ downstream region, in both transfected clones, independent of the H19 RNA level in the corresponding untransfected clones. The CAT activity in the cells transfected with the CAT expression constructs does not therefore bear a direct relationship to the H19 RNA level in the cell. A possible explanation of these findings is that the 5′ upstream and 3′ downstream regions used in our experiments do not contain all the regulatory sequences involved in the control of H19 expression in the JEG-3 cells.

In this work we have also studied the effect of retinoic acid on the activity of the H19 gene promoter. Since retinoic acid has no influence on the H19 RNA level in choriocarcinoma cells, we studied the regions necessary for an increase in the H19 promoter activity by retinoic acid in NT-2 and NCCIT cell lines. These cells are derived from human testicular germ cell tumors and express the H19 gene after addition of retinoic acid to the culture medium (Figs. 3 and 4). In NT-2 cells the level of H19 RNA reaches its peak after 4 days of incubation in the presence of retinoic acid, while growth of NT-2 cells was only slightly slowed down during the first four days of incubation at the time of maximal H19 induction, indicating that H19 RNA accumulation precedes growth inhibition. Under the same incubation conditions, growth was completely inhibited by retinoic acid only after 2.5 weeks. Therefore H19 expression is not the result of growth inhibition. On the other hand, one cannot conclude from these results that H19 expression per se promotes growth inhibition.

A striking finding of this work is that retinoic acid induces not only the 2.3-kb H19 RNA but also the two additional RNAs of ±4.8 and 9.5 kb length which reacted with the H19 probe. These RNAs hybridize with our antisense H19 probe which has a sequence complementary to an 800-bp long sequence upstream of the polyadenylation site of the H19 RNA but do not hybridize with an 819-bp long antisense probe deduced from the −814 to +5 region of the H19 gene. Therefore these RNAs are likely formed by a transcriptional readthrough mechanism using alternative downstream polyadenylation sites.

Retinoic acid may affect the H19 transcription level either in a direct or indirect way, but our results indicate that retinoic acid acts on H19 promoter activity in NT-2 and NCCIT cells in an indirect way. This conclusion is based upon our observation on the slow accumulation of the H19 RNA in the NT-2 cells and, even more strikingly, in the NCCIT cells (Figs. 3 and 5). Therefore it is possible that the effect of retinoic acid is mediated by (a) transcription factor(s) induced by retinoic acid. Likely candidates are transcription factors of the AP-2 family. AP-2.2 was shown to be induced directly by retinoic acid in the mouse embryonal carcinoma cell line P19, the same cell line used in the experiments of Yoo-Warren et al. [14]. Peak levels of the AP-2.2 RNA were detected in mouse P19 cells after 6–12 h [25]. AP.2 seems to be the mediator of the effect of retinoic acid on the expression of the CB-RAP gene in mouse chondrocytes [26]. In NT-2 cells, the cell line we have used in the described study, human AP-2 level was found to reach a peak level 48–72 h after the addition of retinoic acid [27], before the maximal increase in H19 RNA level. In the human H19 5′ upstream region we noted at least 2 sites (around −500 and −40) with sequences nearly identical to the AP-2 consensus motif (a fit of 8 out of 9 nucleotides). Therefore it seems reasonable to assume that a transcription factor of the AP-2 family mediates the stimulation of H19 transcription by retinoic acid.

As most of the sequence of the 5-kb human downstream region (+6 to +11 kb) is unknown we are aware of the possibility that they may contain RARE sequence(s). Our results clearly show that sequences 3′ downstream of the human H19 polyadenylation site cooperate with 5′ upstream regions in the

regulation of the human H19 expression by retinoic acid as was previously described to be the case for the mouse H19 gene by Yoo-Warren et al. [14].

It has also been proposed that retinoic acid can be responsible for post-transcriptional regulation of the expression of a gene such as connexin 43 by altering the stability of its mRNA [28]. Such an assumption, however, cannot explain the results in Fig. 6, showing that retinoic acid does not increase CAT activity in cells transfected by a plasmid carrying the CAT gene under the control of the H19 5′ upstream region alone.

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