SPANDIDOS PUBLICATIONS

A novel large regulator RNA, *B2*, partially overlaps the *HEF1/NEDD9/Cas-L* gene

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Received September 21, 2009; Accepted December 23, 2009

DOI: 10.3892/ijmm_00000420

Abstract. The non-coding RNAs are new players in cellular and molecular biology. Indeed, quantitative and functional non-coding RNA has long been underestimated. There is a great diversity and it seems that much of the genome is transcribed into RNA, while only 1.2% of DNA information is translated into proteins. Non-coding RNA has been categorized according to different specifications so large non-coding RNA includes RNA with 300 to more than 10,000 bp. In this study, we propose a new non-coding RNA named *B2* discovered by differential display. *B2* is a nuclear RNA which is 51,011 bp long with no significant open reading frame. This RNA has a continuous homology with the genomic DNA of the *HEF1/NEDD9/Cas-L* gene located on 6p24-p25. This homology has enabled us to characterize its structure by choosing overlapping fragments to perform several RT-PCRs. *B2* RNA extends from 10 kb upstream of exon 1 of the *HEF1* gene on the 5' end to exon 4 *HEF1* on the 3' end. In addition, a strategic choice of PCR primers enabled us to determine the location of *B2* in the subcellular compartment and then real-time PCR revealed overexpression of *B2* and *HEF1* in certain tissues such as thymus, cervix, liver, and spleen (among the 20 tissues analysed). *B2* seems especially interesting in that it can regulate apoptosis and cell proliferation by modulating *HEF1*. In addition, the fact that cytostatic treatments can induce *B2* reinforces the interest in this new potential target in the development of anticancer treatments. These results show that this novel non-coding RNA is an attractive target.

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Key words: large non-coding RNA, regulator RNA, *HEF1*, *B2*, A190, miRNA, cytostatic molecules

Introduction

The non-small-cell lung carcinomas (NSCLC) represent 80% (1) of all cases of lung cancer but remain very resistant to all therapies currently in use such as chemotherapy, radiotherapy and surgery (2). These therapies focus on fast proliferating cells but such lung cancers have a slow doubling time, sometimes extending for many months, which explains their chemo-resistance. In this context, a new approach would be to develop an appropriate strategy with a greater specificity for cancer cells and a lower toxicity for normal cells thus halting the slowly developing tumour. With this objective, we investigated the screening of original natural or synthetic chemical compounds to find new molecules with innovative mechanisms of action. Cytostatic substances, that induce the arrest of NSCLC cells in the G1-phase of the cycle and then cell death through the apoptosis pathway, should be considered as potential candidates. These selected compounds must also show low toxicity and marked antitumour activity confirmed by good results *in vivo* on nude mice xenografts. Two synthetic molecules, VT1 and A190, have been shown to exhibit all these activities and have already been patented by our laboratory. We have shown in our earlier studies on the NSCLC-N6 cell line that these substances trigger the overexpression of many genes involved in the arrest of G1 proliferation (3). Using the differential display technique and the NSCLC cell line (NSCLC-N6), a large RNA, named *B2*, was identified and located on the human chromosome 6 at the p24-p25 locus. *B2* has no significant ORF (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and one very interesting feature is that it overlaps the *HEF1* gene (4). *HEF1* belongs to a family of docking adapter proteins, including $p130^{Cas}$ and Efs, named the Cas family (5,6). The gene *HEF1* was discovered in 1996 but, in recent years, an increasing number of studies concerning this gene have been published. *HEF1* is involved in many different cellular functions, including migration, mitosis, differentiation and apoptosis (7,8).

HEF1 is a ubiquitous gene, present in many healthy tissues as well as in cell lines at different levels of expression. It is particularly expressed in lung tissue and pulmonary cell lines (5,6,9).

The transcription of *HEF1* is lower in phase G1 and increases in phase S reaching a maximum during phase G2/M. During the cell cycle, the $p105^{HEF1}$ protein and $p115^{HEF1}$, its phosphorylated form, present in phase G1, are cleaved by caspases into $p55^{HEF1}$ and $p65^{HEF1}$ in the G2/M phase (6).

Moreover, *HEF1* is concentrated in the centrosome and stimulates RhoA and Aurora A, two enzymes whose activation/inactivation is essential in the whole mitosis process (10). Dadke and colleagues (19) have shown that *HEF1* is essential, in a very precise quantity, for the cell cycle to progress. The protein depletion resulting from RNA interference, as well as its overabundance, trigger apoptosis. Lastly, during mitosis, $p55^{HEF1}$ associates with the mitotic spindle and decreases suddenly at the end of mitosis as a result of proteosomal degradation.

The major role of *HEF1* in the apoptosis process was first revealed by Law and colleagues (5). They induced *HEF1* over-expression and showed that the cell cycle was blocked in the G1 phase. At the same time, they detected a new form of *HEF1* protein, the $p28^{HEF1}$. This protein is derived from the split of the protein $p65^{HEF1}$ by caspases 3 and/or 7. It leads to cell death by destabilizing the focal adhesion site and stimulating anoikis. To date, the pro-apoptotic activity of *HEF1* has been fully demonstrated and closely linked with the presence of this $p28^{HEF1}$ protein in the cell during *HEF1* overexpression (11).

In the present study, we show that the RNA *B2*, initially isolated through differential display, has a size of at least 51,011 bp and partially overlaps the *HEF1* gene. Furthermore, 10 kb of its sequences are located upstream of exon 1 of *HEF1*. We also report that *B2* has all the structural characteristics of a regulator 'large ncRNA'.

Materials and methods

Cell lines and cultures. Two cell lines were used in this study, A549 and NSCLC-N6 (L16), originating from an adenocarcinoma and an epidermoid lung cancer, respectively. The NSCLC-N6 (L16) is a cell line derived from a human non-small cell lung carcinoma of a previously untreated patient (moderately differentiated classified as T2N0M0) (12). The A549 line was obtained from ATCC (reference CCL-185) (13) and is known to have a wild-type p53 gene while NSCLC-N6 has a mutant p53 gene, so it is very close to tumours *in situ*.

These cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 enriched with 100 IU of penicillin and 100 μg/ml of streptomycin, 2 mM of glutamine and 5% foetal bovine serum. Cell culture plates were maintained in humidified incubators at 37˚C in a 5% CO_2 atmosphere. L16 has a cell doubling time of 48 h *in vitro*, and A549 has a 24 h doubling time.

Extraction of total, nuclear and cytoplasmic RNA. Total RNA was extracted from NSCLC-N6 and/or A549 cell lines using Trizol® Reagent (Invitrogen, CA, USA) and chloroform according to the supplier's recommendations. Cell extracts were centrifuged at 12,000 x g for 15 min at 4˚C. The aqueous phase containing RNA was transferred to a fresh tube. An equal volume of 70% ethanol was added to the aqueous phase and mixed by vortexing. Then, samples were transferred to an RNA spin cartridge supplied with the Trizol® plus RNA Purification Kit (PureLink™ Micro-to-Midi™ Total RNA Purification System, Invitrogen, Applied Biosystems) in order to remove DNA contamination. Then RNAs were eluted with RNase-free water.

The quality and concentration of purified RNAs were assessed using UV absorbance at 260/280 nm and samples were run on 1% agarose gel in order to check their quality. RNAs were stored at -80˚C.

To separate nuclear and cytoplasmic RNAs, some steps were added before the same extraction was carried out. Cells were washed with cold PBS and removed without trypsin and centrifuged for 5 min at 1,000 g. They were resuspended with a lysis buffer (Tris-Cl 50 mM pH 8.0, NaCl 100 mM, $MgCl_2$ 5 mM and 0.5% nonidet P-40) and incubated for a few minutes on ice. Extracts were centrifuged for another 5 min at 13,000 g. Supernatant was collected and pellets were washed twice with PBS. SDS 20% was added to each fraction (supernatant and pellet) and mixed thoroughly by shaking or vortexing. Proteinase K (20 mg/ml) was added and the mix was incubated for 15 min at 37˚C.

DNase treatment. To remove genomic DNA prior to reverse transcription PCR, Turbo™ DNase (Ambion) was used. If the RNA concentration was >200 μg/ml, samples were diluted to 10 μg nucleic acid/50 μl. The 10X turbo DNase buffer was added to sample to a 1X final concentration. Next, 2 U of turbo DNase was added to 10 μg RNA in a 50 μl reaction. Samples were incubated at 37˚C for 30 min. Then DNase Inactivation Reagent was added, the mix was incubated for 2 min then centrifuged at 10,000 x g for 1.5 min. The supernatant, which contained RNA, was transferred to fresh tubes.

RT-PCR. RT was carried out with 1 μg of RNA in 10 μl with 0.125 μg/μl random primers and RNase-free water. This mixture was denatured at 65˚C for 10 min then kept on ice. Subsequently, it was completed to a final volume of 25 μl with a mix containing 8 mM of dNTP mix, 1X of M-MLV 5X reaction buffer, 25 units of recombinant RNasin ribonuclease inhibitor (Promega), and 200 units of M-MLV RT (Promega) and completed with RNase-free water. In order to check whether samples were contaminated by genomic DNA, the same mix was made with RNA without the reverse transcriptase. Following this step, samples were incubated for 2 h at 37˚C. cDNAs were stored at -20˚C. After reverse transcription, cDNAs were used as a template for specific PCR.

B2 was discovered as a 185-bp fragment during RT differential display analysis in NSCLC-N6 cells (4,14). In order to characterize the 5' and 3' ends, RACE-PCR was first applied. Then DNA homology with the human chromosome 6 (GenBank No. AL 136139.6 and AL 139807.6 sequences) was exploited to perform RT-PCR. Primers were chosen so that two contiguous amplicons overlapped by at least 100-150 bp (Fig. 1). All primers were designed using the Primer3 software (http://frodo.wi.mit.edu/cgibin/primer3/primer3_www.cgi).

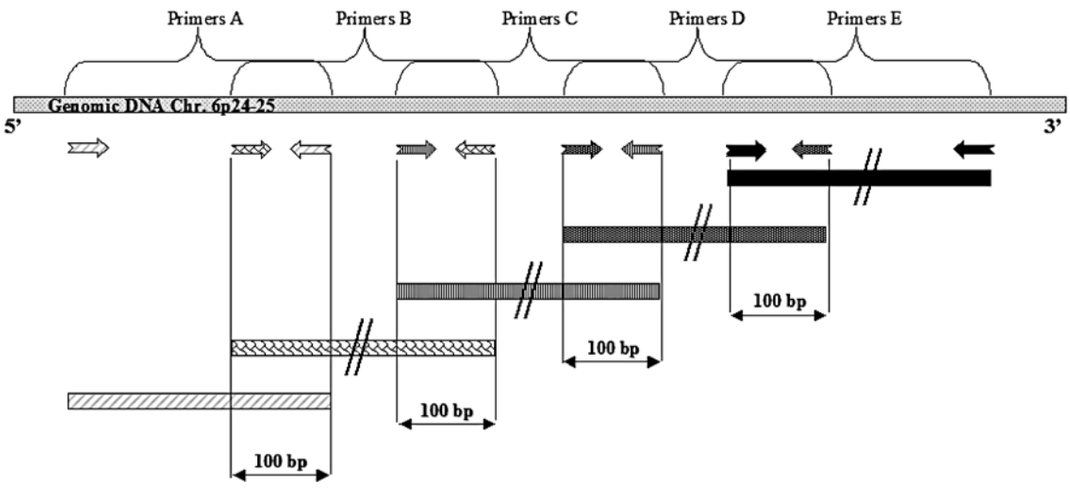

Figure 1. Schematic strategy for the identification of the *B2* sequence. The genomic DNA sequence of chromosome 6 was used for sequence primers designed with the website Primer 3. Each amplified fragment was 1 kb long and overlapped the upstream and downstream amplified fragment over about 100 bp.

Table I. Characteristics of the probes used.

Probe name	Probe sequence	Tm[a] (˚C)	Product size (bp)
Left primer B2-EX1-04	5'-AGGCCGCAAGACTCATCTTA-3'	60.5	533
Right primer B2-EX1-04	5'-GGATGCACTCCAACCAGACT-3'	59.9	
Left primer B2-Si3	5'-CAACTTGGGGTGCATTCTTC-3'	60.5	1,081
Right primer B2-Si3	5'-CTGAGAACCAGCATGATGACA-3'	59.9	
Left primer ß2-M EX	5'-CCGATATTCCTCAGGTACTCCA-3'	60.0	931
Right primer ß2-M EX	5'-ACATGTCTCGATCTATGAAAAAGA-3'	60.0	
Left primer ß2-M INT	5'-AGGCTATCCAGCGTACTCCA-3'	60.0	320
Right primer ß2-M INT	5'-CCTCCATGATGCTGCTTACA-3'	60.0	
Left primer HEF1 Exon 5	5'-AGCAGGGCTTAGGGAAAAAG-3'	60.0	1,218
Right primer HEF1 Exon 5	5'-GCCAAGATATTCAGGGACCA-3'	60.0	

[a]Tm, temperature

Among all the *B2* primers, B2-Si3 (Table I) matched the genomic sequence located between exons 2 and 3 of the *HEF1* gene and B2-Ex1-4 matched 4,000 bp upstream of the *HEF1* exon 1. Both were used to assess tissue expression and subcellular location.

HEF1 Exon5 primers (Table I) were used to assess *HEF1* expression on the Firstchoice Panel®, ß2-M-INT and ß2-M-EX were used as controls for ß2-microglobulin amplification. These primer pairs were as follows, left B2-M-INT matched on the junction of the genomic sequence before exon 2 and exon 2 of the gene were included, and right B2-M-INT on the junction of the genomic sequence before exon 3 and exon 3 were included. They were employed for specific nuclear amplification of ß2-microglobulin RNA.

Left ß2-M-EX matched on the junction exon 1-exon 2 and right ß2-M-EX on the junction exon 3-exon 4. They were used for cytoplasmic and nuclear amplification of ß2-microglobulin RNA.

The PCR mix, consisting of 1X final of CoralLoad PCR buffer (Quiagen), 200 μM of each dNTP, 0.5 μM of right Primer, 0.5 μM of left Primer and 2.5 units/reaction of Taq DNA polymerase (Quiagen) was in a final volume of 25 μl. The amplifications were performed with the following protocol, 3 min at 94˚C for initial denaturation then 39 cycles of amplification (60 sec at 94˚C, 60 sec at 60˚C for primer annealing, 60 sec at 72˚C for elongation), and 7 min at 72˚C for final elongation. PCR products were analysed on 1% agarose gels stained with ethidium bromide (BET).

Race-PCR. The first step of this experiment consisted of a specific reverse transcription. It was achieved from tRNA extracted from A549 and L16. RNA (1 μg) was used with 1 μM of a specific primer (sequence) in 8.5 μl final volume. The mix was denatured for 10 min at 65˚C in a thermocycler. Then, the 5X reaction buffer (Promega), dNTPs (10 mM), 20 IU RNase inhibitor (Promega), 100 mM DDT, 200 IU MMLV RT in 20 μl final volume were added and incubated at 37˚C for 50 min, then 5 min at 70˚C. In the second step, a poly C was added to the 3' end. The reaction buffer 5X, dCTP 2 mM and 10 U/μl were added to the previous mix and incubated for 15 min at 37˚C and then at 70˚C for 10 min. The third step is named anchored PCR. It is a normal PCR

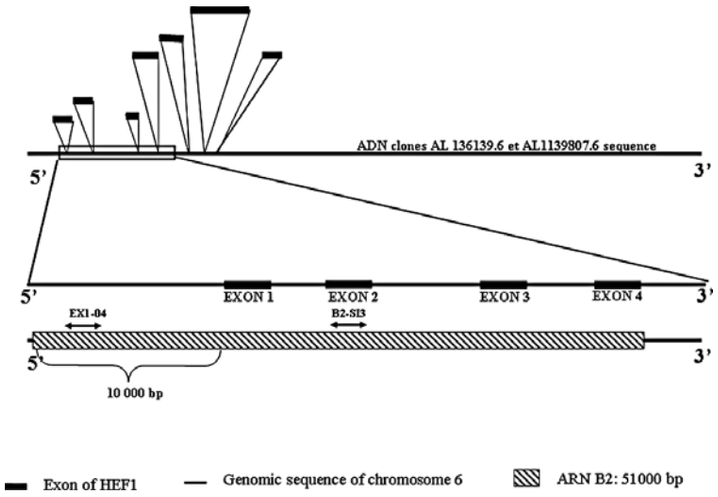

Figure 2. Schematic representation of homology between *HEF1* and *B2* in the region 6p24-25. *B2* is a large ncRNA that originates 10 kb upstream of the first exon of *HEF1* and ends at the intronic region encompassing exon 4 of *HEF1*. Specific primers of *B2* are shown in the figure. B2-SI3 spans the genomic sequence located between exons 2 and 3 of HEF1 and EX1-04 is located upstream of exon 1 of *HEF1*.

but there is only one primer (sequence) and oligo dG 10 μM is different from a classic PCR.

RNA panel. The RNA panel was used first in RT-PCR and then in qRT-PCR. RT-PCR was performed to study the expression of the *B2* RNA in different tissues. The First Choice® Human Total RNA Survey Panel (Ambion) was used as a source of RNA from 20 different normal tissues (ovary, skeletal muscle, trachea, prostate, placenta, thymus, small intestine, bladder, testes, brain, colon, esophagus, kidney, spleen, adipose, liver, heart, thyroid, cervix, lung). B2-EX-1, B2-SI3, *HEF1*, and *ß2-microglobulin* (ß2-M EX and INT) amplifications were each carried out with all 20 RNAs.

Then these 20 normal tissues were tested in real-time PCR. *B2* and *HEF1* amplifications were carried out following the instructions of the manufacturer of SYBR Green PCR mix (Applied Biosystems) on the ABI Prism® Sequence Detection System 7300. The primers were designed using Primer 3 (as described above). For the gene *HEF1*, the primers were chosen on two different exons (1 and 2) to be sure of no interference between the amplifications of *HEF1* and *B2*. The primer sequences were HEF1, forward 5'-CGCT GCCGAAATGAATAT-3', reverse 5'-CCCTGTGTTCTGCT CTATGACG-3'; *B2*, forward 5'-ATTCCCTTGGATCTTGC CTT-3', reverse 5'-CGTGAGGTCTGCCACTAC-3'. The relative expression was normalized with ß-actin added to each gene, forward 5'-ATTCCCTTGCCTTCTTGGAT-3', reverse 5'-CGTGAGGTCTGCCACTACAG-3'. A control sample was used for each PCR to check the genomic DNA contamination of the cDNA template. The results were analysed using GenAmp 7300 SDS (Applied Biosystems).

Results

B2 RNA is at least 50 kb. From the 185 bp long initial fragment, we carried out as many as 117 RT-PCR (sequences not shown) to characterize the whole sequence of the *B2* RNA. Each amplicon was a 1-kb fragment and overlapped the previous and the next over at least 100 to 150 bp (Fig. 1).

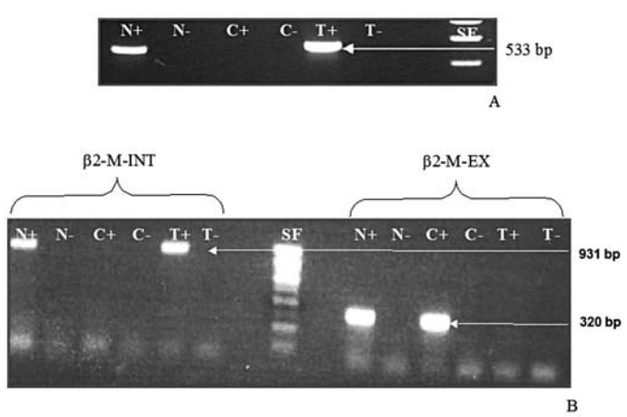

Figure 3. PCR amplification analysed on agarose gel 1% stained by BET. Cytoplasmic RNA and nuclear RNA were analysed by RT-PCR with *B2-EX1-04* primers (A) and control primer (ß2-M-INT and ß2-M-EX) (B). (A) nuclear fraction with (N^+) or without (N^-) reverse transcription; cytoplasmic fraction with (C^+) or without (C^-) reverse transcription; T^+ genomic DNA, T^- negative control; SF. (B) nuclear fraction with (N^+) or without (N^-) reverse transcription; cytoplasmic fraction with (C^+) or without (C^-) reverse transcription; T^+ genomic DNA, T^- negative control. First six lanes show the amplification with ß2-M-INT and the last six lanes with ß2-M-EX.

To date, considering all the fragments joined together, *B2* spreads over at least 51,011 bp. It shows a total homology with the region 6p24-p25 of the human genome without any interruption. So it is formed with a single large exon.

B2 overlaps one part of the *HEF1* gene, 10 kb match upstream of the exon 1 *HEF1* and the last 40,000 bp spread from exon 1 to exon 4 of *HEF1* (Fig. 2). No significant ORF was identified in the whole sequence. Finally, the 5' end is defined. The sequence was published in Genbank, GQ497714.

B2 is a nuclear RNA. RT-PCR was performed on both nuclear and cytoplasmic RNAs. B2-Si3 as well as B2-EX-1 primers show amplification only on the nuclear RNA extract (Fig. 3). B2-Si3 lies on the initial differential display located in the

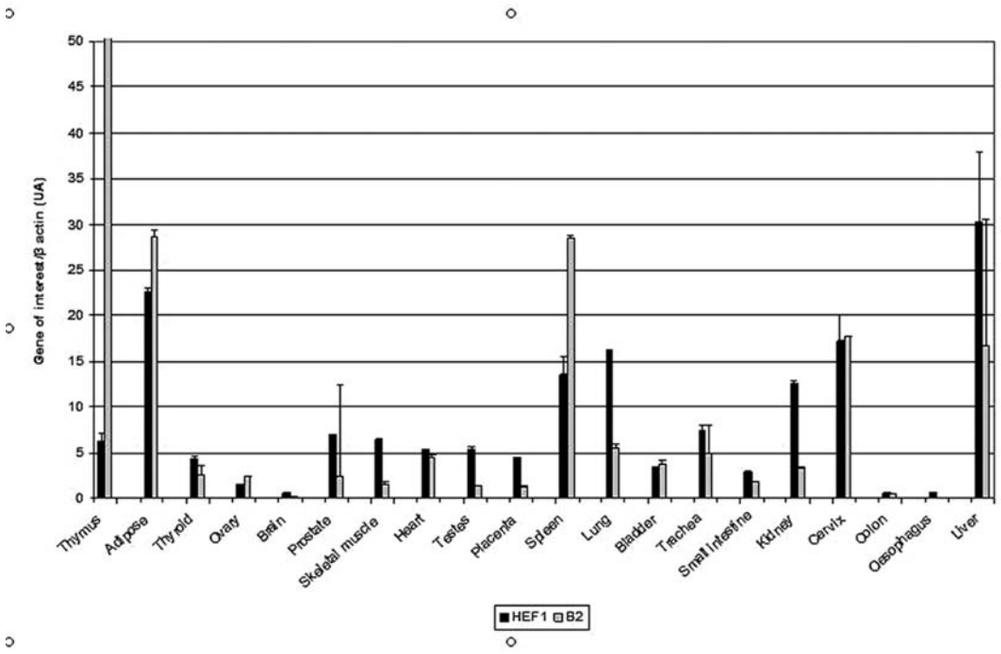

Figure 4. Expression of the gene *B2* (grey) and *HEF1* (black) on FirstChoice® Human Total RNA Survey Panel (Ambion) by real-time PCR.

genomic sequence between exons 2 and 3 of the *HEF1* gene but B2-EX4-1 matches the part upstream of exon 1 of *HEF1*, thus ruling out any interference with *HEF1* mRNA. We have shown only the result with B2-EX4-1 because it is the same result.

Then RT-PCR was carried out with ß2-M-EX and ß2-M-INT primers on the same RNA extracts and the same cDNA. As might be expected, PCR amplification with ß2-M-INT was possible only on the nuclear RNA extract. This experiment, with the DNase treatment, confirms that nuclear RNA could actually be amplified in our extracts. Moreover, it also confirms that no nuclear contaminants were present in the cytoplasmic RNA fraction. With ß2-M-EX, PCR fragments were present both in the cytoplasmic and in the nuclear fraction because of the mRNA maturation that occurred in the nuclear compartment. Moreover, these latter primers enabled us to validate the RT step.

B2 RNA is as ubiquitous as the HEF1 gene. As *B2* overlaps the *HEF1* gene, we were interested in the relation between these two genes. More precisely, we investigated whether *B2* and *HEF1* were expressed in the same tissues. To test this, we chose to use the same commercial RNA panel that had been employed in a recent study on *HEF1* (9). So additional RT-PCR was performed with B2-Si3 and B2-EX1-4 primers. Each primer pair was tested on all 20 RNA extracts.

The expression of *B2* RNA could be amplified in all normal tissues tested, such as for *HEF1*. The expression of these two genes were analysed with real-time PCR.

Quantification of B2 RNA and the HEF1 gene. As these 2 genes could be amplified in the 20 tissues tested, we investigated this directly using quantitative RT-PCR to analyse *B2* and *HEF1* expression in mRNAs prepared from 20 human tissues (Fig. 4). *HEF1* was most abundant in adipose, lung, cervix, liver and spleen and was detected at lower levels in the other tissues while *B2* was most abundant in thymus, adipose, cervix, liver and spleen and was detected at lower levels in the other tissues (Table II). These results are consistent with the study of Golemis *et al* (9).

Table II. *B2* and *HEF1* amplification from different tissues of the FirstChoice® Human Total RNA Survey Panel.

Tissue (+)	Expression *B2*	Expression *HEF1*	Tissue (x)
Bladder	+ x	+ x	Skeletal muscle
Testes	+ x	+ x	Heart
Oesophagus	+ x	+ x	Cervix
Brain	+ x	+ x	Lung
Trachea	+ x	+ x	Spleen
Colon	+ x	+ x	Kidney
Placenta	+ x	+ x	Small intestine
Thymus	+ x	+ x	Liver
Thyroid	+ x	+ x	Adipose
Ovary	+ x	+ x	Prostate

Discussion

Since the sequencing of the entire genome, we know that the transcriptome is much bigger than had previously been supposed. Particularly, in recent years numerous studies have shown the importance of small non-coding RNAs such as miRNA, small nuclear and nucleolar RNAs, in the regulation of gene expression. However, a new class of non-coding RNAs has also now emerged which comprises RNAs of several thousand bp. Most of these 'large RNAs' are not spliced and

are at least 10 kb in size. Their function is not yet clear but some are involved in the regulation of the expression of adjacent genes. Among the best known is *Air*, a non-coding unspliced RNA of 108 kb which acts as the 'silencer' for three genes coding for proteins of the igf2r cluster (15). Another is NTT, an RNA of 17 kb situated in 6q23-q24 next to the receptor γ of interferon, whose expression it regulates in $CD4^+$ cells. Finally, there is Xist, a nuclear RNA of 17 kb which is involved in the transcriptional silencing of one of the two X chromosomes in the female embryo (16).

The present study revealed a new large non-coding RNA, called *B2*, which is 51 kb long. It is nuclear, has no open reading frame and is found in the p24-p25 region of chromosome 6. It is particularly interesting because it presents a total and continuous homology with genomic DNA, and thus consists of one large exon that overlaps the *HEF1* gene from exon 4 to >10,000 bp upstream of exon 1. In addition, we have compared the tissue expression of *B2* and *HEF1* in 20 RNAs of healthy tissues. Our results show that these two molecular targets are expressed in all the healthy tissues of the panel. However, *HEF1* is overexpressed in adipose, spleen, cervix, liver, and lung. Moreover, when the expression of *B2* is weaker, as in liver, there is an overexpression of *HEF1*. These results are in agreement with those published by Golemis and co-workers who showed an overexpression of *HEF1* in lung (9). *B2*, however, is overexpressed in adipose, spleen, cervix, liver and thymus (Fig. 4). In fact, in the thymus, *B2* is greatly overexpressed compared to *HEF1*. Collectively, the results show that overexpression of *B2* leads to the repression of the *HEF1* gene, as observed in the thymus, adipose, and spleen. Conversely, when *B2* is weakly expressed, *HEF1* is overexpressed as seen in the lung and kidney. These results confirm a link between the overexpression of *B2* and the repression of *HEF1*, which implies that the non-coding RNA *B2* regulates the *HEF1* gene.

This could occur by numerous mechanisms, identified to varying degrees in previous studies. Among the best known is transcriptional interference. This results from a strong promoter acting on a weaker promoter located downstream and thus affects two genes close together (17). Another mechanism can explain this regulation of *HEF1* by *B2*; due to its nuclear location and large size, *B2* can induce a loop in the chromatin enabling the repression of *HEF1*. Such chromatin loops have already been suggested to explain how much ncRNA takes part in the phenomenon of higher-order chromatin silencing (17). Alternatively, the transcription of such a large RNA could simply enable all the transcription factors to be sequestered thus preventing gene *HEF1* transcription (17). Other hypotheses are also feasible; *B2* is a non-coding nuclear RNA which could form an RNA/DNA triple helix during the transcription of the RNA of *HEF1* thus blocking it as has already been shown (17). The final hypothesis to be considered here is that *B2* acts as an RNA precursor of miRNA. In fact, according to one study, *H19* was one of the first non-coding RNAs to be identified and has recently been described as a precursor of miRNA (18). As *B2* RNA overlaps several exons of the *HEF1* gene, it seems possible that *B2* could induce miRNA directed against *HEF1* mRNA. Moreover, bioinformatic analysis shows that the sequence of the *B2* gene can generate numerous sequences typical of miRNA. These sequences would be recognized and cleaved into pre-miRNA by Drosha in the nuclear compartment then transferred by exportin 5 into the cytoplasm where the Dicer complex would transform these pre-miRNA into miRNA (18).

The *HEF1* gene is known for its involvement in the control of cell growth, in apoptosis and in cell motility through its interaction with cellular adhesion sites and the mitotic spindle (5,19). *HEF1* is responsible for the production of 4 different proteins, $p115^{HEF1}$ and $p105^{HEF1}$ cleaved into $p65^{HEF1}$ and $p55^{HEF1}$ by caspases. Moreover, Law *et al* showed that overexpression of *HEF1* in MCF-7 cells increased the proteolytic activity of caspase 3 and/or 7 leading to the cleavage of $p65^{HEF1}$ into $p28^{HEF1}$ (20). The C-terminal region of this $p28^{HEF1}$ seems to be responsible for the pro-apoptotic activity of *HEF1*. In fact, $p28^{HEF1}$ favours the cleavage of Focal Adhesion Kinase (FAK) leading to apoptosis (11).

Cell proliferation, motility and growth are the three target areas of anticancer chemotherapy. In this approach, our aim was to demonstrate that *HEF1* and *B2* are involved in halting proliferation by inducing apoptosis in the anticancer chemotherapy of lung cancer. The work of Moreau *et al* (20) has highlighted the antitumour effect of the cytostatic molecule A190 on nude mice xenografted by non-small cell lung carcinomas.

The results that we have obtained on the two lung cell lines A549 and L16 during treatment with A190 confirm the existence of *HEF1* gene regulation by *B2* RNA.

A190 induces cell apoptosis at the same time as it increases the expression of both *HEF1* and *B2* RNA. This overexpression occurs after 60 h of treatment and falls dramatically after 70 h. If non-coding *B2* RNA acts as a precursor of miRNA, it would enable the production of miRNA directed against *HEF1* mRNA, explaining the sudden drop in the level of expression. The decrease in *HEF1* mRNA would lead to a lack of *HEF1* protein which would fall below the minimal threshold required for the cell cycle to progress and apoptosis would be triggered.

The team of Golemis (7,8) has demonstrated that the normal progression of the cell cycle requires a precise quantity of the HEF1 protein. This must lie between an upper and a lower limit, thus three possible situations can occur. If there is the correct quantity of HEF1 protein in the cell, the truncated form $p55^{HEF1}$ contributes to the normal cell cycle and mitosis can take place. However, if there is excess HEF1, above the upper limit, then the $p65^{HEF1}$ form, also in excess, is cleaved into $p28^{HEF1}$ which initiates apoptosis. Similarly, if there is insufficient HEF1, below the lower limit, the cell cycle will be irreversibly stopped in the very early stages of mitosis.

The latter situation would be applicable during treatment of cells A549 and L16. This would explain how the molecule A190 could trigger apoptosis even in the absence of $p28^{HEF1}$ which has never been detected by Western blotting (data not shown).

There remains a great deal to discover about large ncRNAs. Their study is at an early stage and it would not be surprising to see their importance grow in numerous diseases, particularly in cancer. Our identification here of such an RNA in lung cancer cells is one illustration. *B2* seems especially interesting in that it can regulate apoptosis and cell proliferation by

ng *HEF1*. In addition, the fact that cytostatics can induce *B2* reinforces the interest of this new potential target in the development of anticancer treatments.

Acknowledgements

We thank La Ligue Départementale Contre Le Cancer, Région Pays de La Loire and Professors P. Tsits and Antoniadou-Vyza and Anne-Laure Biang for their contributions.

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