The second intron of the human galectin-3 gene has a strong promoter activity down-regulated by p53

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Abstract Galectin-3 is a galactose-specific lectin which has been shown to be involved in several biological functions such as cell growth regulation, cell aggregation and cell differentiation. The partial cloning of the human genomic sequences reveals the presence of a 651 bp intron, 18 bp downstream of the translation initiation site. This intron contains several regulatory elements found in many eukaryotic genes. This sequence, when inserted upstream of a promoter-free luciferase gene, induces the expression of luciferase, demonstrating the promoter activity of the intron upon transfection in human or murine cells. This promoter activity is down-modulated by wild-type p53 but not by a mutated form of p53.

Key words: Internal promoter; Intron; Polymerase chain reaction; Soluble galactose binding lectin

1. Introduction

Galectin-3 [1], a soluble galactose binding lectin, has been identified independently by different laboratories. On the basis of cDNA sequencing, all galectins-3 are closely homologous, namely CBP35 from 3T3 fibroblasts [2], L-31 and L-34 from human and murine tumour cells [3,4], Mac-2 from human and rat macrophages [5,6], Ig-E binding protein or ∈BP from basophilic leukaemia cells [7,8], and L-29 in human and rat lung [9,10]. The protein can be found intracellularly in the cytosol and in the nucleus and extracellularly in the medium and at the cell surface [3,11–14], suggesting its involvement in various biological processes. The lectin expression changes during development [15], is modulated by serum stimulation and is dependent on the cell growth status [16,17]. The lectin expression is also increased upon oncogene or viral transformation [4,11,18,19] and is high in a wide range of human and murine tumour cells [11,20]. The murine L-34 could play a role in metastasis as an active component in the progression of tumour to metastatic cells [4,21]. In addition, this lectin, as an IgE binding protein, may also be involved in allergic disorders [8,22]. In macrophages, the lectin is elicited by inflammatory stimuli and is regarded as a differentiation marker [5].

So far, little is known about the regulation of lectin gene expression. We undertook the determination of the genomic sequences corresponding to the 5′ moiety of the human galectin-3 gene using the ligation mediated-polymerase chain reaction (LM-PCR) [23]. We describe the isolation and characterization of an intron located within the coding sequence 18 bp downstream of the translation initiation ATG codon. Its sequence reveals several regulatory elements known as consensus sequences specific for various transcription activators of eukaryotic genes. Furthermore this intron, when inserted into a plasmid containing a promoter-free reporter gene, elicits a strong promoter activity which is, in addition, down-modulated by wild-type p53 but not by mutated p53.

2. Materials and methods

2.1. Cloning of genomic sequences by ligation mediated PCR

10 μg of human placental genomic DNA were digested with the restriction enzyme *Hin*dIII (New England Bioloabs, MA) and ligated to 6 μg of pUC18 plasmid DNA previously cut with *Hin*dIII and *Eco*RI. 1 μg of DNA of the ligation reaction was used as the template for PCR which was carried out in a DNA Thermocycler (Perkin-Elmer-Cetus, Norwalk, CT) in a reaction mixture containing 1.25 U of *Taq* DNA polymerase (Promega Corp., Madison, WI) and 1 μM of each PCR primer in 50 μl 1× *Taq* polymerase buffer. The PCR primer MN-11, 5′-AGTGCCACCTGACGTCTAAG-3′, is complementary to a sequence on pUC18 (nt 2608–2628); the PCR primer MN-39, 5′-GTAGCCCCTGCCCAGCAGGCTGGTT-3′, is located 82–108 bp downstream of the ATG translation initiation codon of the human galectin-3 cDNA [4,6,8] (Fig. 1). Thirty amplification cycles were performed (1 min denaturation at 94°C, 2 min annealing at 55°C and 3 min extension at 72°C). 2 μl of the reaction were used as the template for a second round of PCR using two nested primers M13F, 5′-GTAAAACGACGGCCAGT-3′, located on pUC18 (nt 379–395) and GAL67, 5′-ATCCTTGAGGGTTTGGGTTT-3′, located 45–64 bp downstream of the ATG translation initiation codon on the galectin-3 cDNA [4,6,8]. 1 μl of this second PCR reaction was used for ligation with a linearized pCR1000 plasmid vector and transformation of *Escherichia coli* as recommended by the manufacturer (TA cloning system; Invitrogen Corp., San Diego, CA). Colonies were probed with GAL25, 5′-ATGGCAGACAATTTTTCGCTCC-3′, an oligonucleotide internal to GAL67 and located from nt 1 to 22 on the galectin cDNA. Positive plasmids were sequenced on both strands using Sequenase (United States Biochemical, Cleveland, OH).

2.2. Construction of reporter plasmids and transfections

The PCR fragment was excised from the pCR1000 plasmid after cleavage with *Not*I and *Hin*dIII and introduced at the same sites into pGEM11Z(f−) (Promega Corp., Madison, WI). The luciferase reporter gene was isolated from pOLUC [24] by *Bam*HI and *Sac*I and introduced downstream of the intron into the plasmid at the same sites to produce the expression vector pGEM99-LUC. Insertion of the PCR fragment, in reverse orientation with respect to the luciferase gene, produced the plasmid pGEM99R-LUC. Deletion of the PCR fragment in pGEM99-LUC produced the promoter-free luciferase gene vector pGEM-LUC used as a negative control. A positive control was obtained by transfection with pSV2LUC, a plasmid in which the luciferase gene is driven by the SV40 early region promoter [24]. These vectors were transfected, according to Midoux et al. [25], into human osteosarcoma HOS (American Type and Culture Collection CRL 1543, Rockville, MD) and murine macrophages J774 cells clone E [26] which express the mRNA specific for the lectin. Briefly, 10^6 cells were incubated for 4 h in a medium containing 100 μM chloroquine and 5 μg of plasmid DNA complexed with 12.5 μg glycosylated poly-L-lysine. 48 h later, luciferase activity was assayed, using an automated luminometer

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(Berthold Lumat LB 9501; Colombes, France). Co-transfection experiments were performed by using 5 μg of pGEM-99LUC and 0.5 μg of pBR322 or of p53SN3 or of p53SCX3; these two last vectors encode for wild-type or mutated p53, respectively [27].

2.3. cDNA synthesis and amplification with PCR (RT-PCR)

2 μg of total cell RNA, extracted from HOS cells by the guanidium-phenol-chloroform method [28], were reverse transcribed in a reaction mixture containing 30 U AMV reverse transcriptase (Promega Corp., Madison, WI), and 200 μM of the primer GAL764, 5′-TCTGCCCC-TTTCAGATTATATC-3′, located from nt 747 to 768 at the 3′end of the galectin-3 cDNA [4] in 20 μl reaction buffer for 2 h at 42°C. One-tenth of the reaction was used as the template for 30 PCR cycles initiated with a 3′ primer MN-38, 5′-TCTGTTTGCATTGGGTTC-ACC-3′, located upstream of GAL764 from nt 411 to 432 on the cDNA and GALi316, 5′-TTCTGATGTTTGTGATTGTTTTTC-3′, located from nt 317 to 340 in the cloned PCR fragment (Fig. 2). A second round of PCR was performed using one-fiftieth of the first PCR product.

3. Results

3.1. Cloning of genomic DNA by ligation mediated-PCR

The genome walking scheme is shown in Fig. 1. Human genomic DNA was digested with different enzymes and ligated onto the linearized vector pUC18. The resulting ligation products were amplified by using primers MN-11 and MN-39 which lie on the cloning vector and on the galectin-3 gene, respectively. Upon gel electrophoresis, the amplified products appeared as a smear with few discrete bands barely visible (not shown). In order to enhance the specific amplification, a second round of PCR was performed with two nested primers, M13F and GAL67, which are located on the cloning vector and on the 5′ part of the coding region of the cDNA, respectively. A unique band of about 800 bp was visualized by gel electrophoresis (not shown). This PCR fragment was inserted into the pCR1000 vector. Positive clones were screened with GAL25, an internal primer, and sequenced.

3.2. Characterization of the human genomic sequence around the translation initiation site

The expected PCR fragment should have at one end the M13F primer from pUC18 DNA followed by the *Hin*dIII cloning site (Fig. 1) and at the other end the GAL67 primer. The sequence, reported in Fig. 2, has indeed such a structure. The exact size is 778 bp in good agreement with the gel electrophoresis data. Upstream of the PCR primer GAL67 the first 26 bp are identical to those of the published sequence of the cDNA (GenBank accession numbers J02921, M35364, M36682, M64303) but further on the sequence of the PCR product diverges from that of the cDNA; the 26 bp sequence contains the four last 3′ bases of GAL25, the oligonucleotide used as a probe. The remaining 18 bp of GAL25, which contains the ATG translation initiation codon, were found 63 bp downstream of the 5′ end in the PCR fragment, indicating that an intervening sequence of 651 bp is located within the sequence of GAL25. Consensus sequences for 5′ and 3′ splicing sites of the intervening sequence are present, confirming this assumption. Four bp upstream of the ATG translation initiation codon the sequence of the PCR fragment diverges again from the cDNA indicating the presence of another intervening sequence. Thus, the ATG translation initiation codon is contained within a short 22 bp exon. This structure in the 5′ end of the coding sequence appears to be identical to that of the murine galectin-3

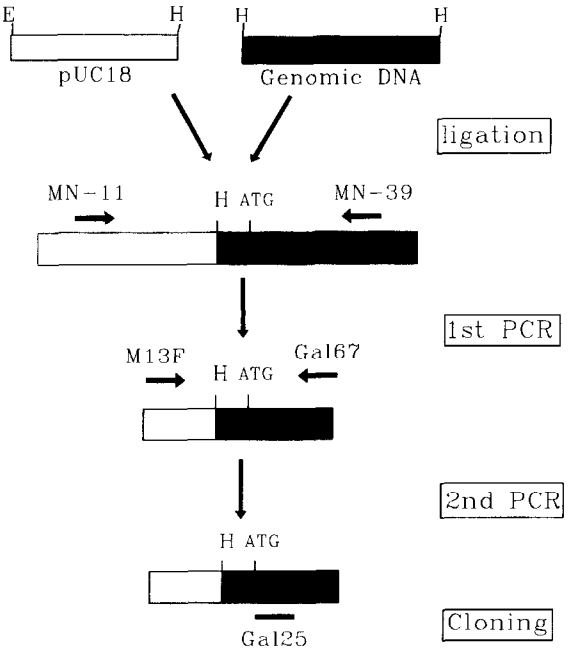

Fig. 1. Experimental design for cloning of genomic sequences by LM-PCR. Human genomic DNA (black boxes) digested with *Hin*dIII (H) was ligated to pUC18 (open boxes) previously double digested with *Hin*dIII and *Eco*RI (E) in order to avoid self ligation. PCR was initiated with two primers located on pUC18 (MN-11) and downstream of the ATG translation initiation codon site of the galectin-3 gene (MN-39). A second round of PCR was initiated with nested primers (M13F and GAL67) in order to specifically amplify the relevant sequences. The PCR product was inserted into pCR1000 and clones were screened with GAL25, an internal primer located at the 5′ end of the coding sequence of the gene of galectin-3 cDNA.

gene [29]. Consequently the incomplete intervening sequence at the 5′ end of the PCR fragment represents very likely the first intron of the human galectin-3 gene, the 22 bp exon is the second exon and the 651 bp intron is the second intron.

Consensus sequences specific for transcription factors AP-1, AP-2, GC box, NFκB, CTF, p53 and CCAAT which are present in the 651 bp intron, are indicated in Fig. 2.

3.3. Promoter activity of the cloned intron from the galectin-3 gene

In order to assess the putative promoter activity of the 651 bp isolated intron, the PCR fragment was subcloned upstream of a promoter-free luciferase gene to produce the pGEM99-LUC plasmid, which was then transfected into human osteosarcoma HOS cells and murine macrophage J774 clone E cells. The luciferase activity of these two cell types upon transfection with the pGEM99-LUC plasmid was quite high (Fig. 3), indicating a strong promoter activity of the sequence isolated from the galectin-3 gene. The activity of luciferase is lower in J774 clone E cells than in HOS cells; this reflects a difference in the transfection efficiency of these two cell types (unpublished results). The luciferase activity in HOS cells was very high, around 700,000 RLU with either promoters: the SV40 early region or the second galectin-3 gene intron. In the monocytic line J774 clone E, the second galectin-3 intron used as a promoter led to a luciferase activity slightly higher (about twice) than when the luciferase gene was under the control of the SV40

M13F		HindIII				
gtaaaacgac	ggccagtgcc	aagctt				
		3'ss		GAL25	5'ss	
TTAGGATAAA	ATGATAA*TCT*	*TTGTTTCTTT*	*CAGGAAAATG*	GCAGACAATT	TTT*CGGTAAG*	5
	CTF					
*T*GTTTTATGC	CTGTTTCTTC	CCCTTGATCA	GCTCCACATG	GTTGAGGGTT	GGGGGTTTTG	65
	NFkB					
TTTTTACCAT	GACTTTCCCT	TTTCACTCTC	CCACTGCGTG	CTTCCCCTGG	ACTCATTTGT	125
CAAT		p53				
CCAATGAGGG	CTTGCAAGCT	GGAGCCTTGT	TTTTCCAGCA	GCAGATTTGG	GAAGAAAGCC	185
					AP-2 GC box	
AGGCAGAGCG	AGCCTGGGA	CTCACTCACA	GTAACCCTTT	CACCAAAAGG	CCCAGGGCGG	245
	AP-1					
AAGGGAGTGG	ACTCTGCCGG	CAGGAGCTGA	GAAATCCTCT	GAGTAGCGGG	AAGTGCGGAT	305
	GALi316					
CAGTCTGGGC	ATTCTGATGT	TTGTGATTGT	TTTTCTCACG	GTGATGAAAA	AGTATGTGCT	365
ATAAGTAGAG	AGCGCTAACT	CCTGACTTGA	GCTAATTATG	AAAATGCAGC	CCTCCCTGAT	425
CTGAGACGTT	GGGAGGCAAG	AATAAAGTGA	AAAAGTATAT	GTAATCCCAA	CATCTAATTT	485
TAGTCTTAGA	AACTCAAACT	ATTAATAAGT	GGAAAAAGTT	TAATGATATG	CATGTAATGC	545
CTTTGCCATA	TTCCTCTCCT	TCTTAGATCA	CATATTCCTA	TTTTCCTGAA	AATTCTGCTT	605
			3'ss	GAL25		
TTGAGAATGC	TTTCTGTCCC	GTAATTGTGT	ATG*TCTTTCT*	*TTCCAGCTCC*	**ATGATGCGTT**	665
		GAL67				
ATCTGGGTCT	**GGAAACCCAA**	**ACCCTCAAGG**	**AT**			

Fig. 2. Nucleotide sequence and regulatory elements from the PCR fragment containing a complete intron of the galectin-3 gene. The nucleotides in lower case letters represent the sequence from the pUC18 plasmid from primer M13F up to the *Hin*dIII cloning site. The bold nucleotides represent the sequence of the published cDNA. The sequence located downstream of the *Hin*dIII site and upstream of the GAL25 primer is the 3′ end of an incomplete intron and the sequence downstream the GAL25 represents a complete 651 bp intron. The ATG translation initiation codon is located at the 5′ end of the GAL25 primer. Italics represent consensus sequences for 5′ and 3′ splicing sites of intron sequences (named 5′ss and 3′ss). Putative transcription elements are underlined (AP1, NFκB, CTF, AP-2, CCAAT and GC box). The consensus binding site for p53, consisiting of two 10 bp motifs [27], is stippled. Doubly underlined nucleotides mark the sequences of the oligonucleotides GAL25 used as a probe after cloning the PCR fragment, GAL67 used as PCR primer and GALi316 for detection of transcripts by RT-PCR. Nucleotides are numbered relative to the first base of the 651 bp intron. This sequence has been submitted to the GenBank/EMBL Data Bank with accession number U10300.

early region. When the second galectin-3 intron was inserted in reverse orientation (pGEM99R-LUC), the luciferase activity was very low, about 15 times lower than that obtained with pGEM99-LUC (Fig. 3A).

Because the second galectin-3 intron contains a putative p53 binding site [30], we were interested to know whether the internal promoter activity could be sensitive to p53 status. HOS cells were co-transfected, in eight independent experiments, with pGEM99-LUC as above and with pc53SN3, a plasmid encoding wild-type p53 [27]. Under such conditions, the luciferase activity was 8–10 times lower than that obtained after co-transfection with pBR322 in place of pc53SN3. This down-modulation was not observed when pc53SCX3, a vector encoding a mutated form of p53, was used. Addition of a β-galactosidase encoding vector revealed no changes in the efficiency of transfection when wild-type or mutated p53 plasmids are used, indicating that the down-modulation effect is not the result of differential DNA incorporation (data not shown).

3.4. *Detection of transcripts containing sequences from the isolated intron of galectin-3 gene in HOS cells*

Reverse transcription was performed on mRNA from HOS cells initiated with the primer GAL764 located at the 3′ end of the coding strand of the cDNA (Fig. 4). PCR was subsequently performed using the 3′ primer MN-38 located upstream of GAL764 on the cDNA and the 5′ primer GALi316 located on nucleotides 316–339, in the cloned PCR fragment (Figs. 2 and 4). A 740 bp fragment of the excpected size was detected following the two rounds of 30 PCR cycles. Its specificity was further checked by DNA sequencing (data not shown). This amplified fragment does not arise from contaminating genomic DNA because no amplification could be evidenced from PCR performed on non-reverse transcribed RNA or from amplification of the pre-mRNA because three additional intervening sequences are located between the second intron and the GAL764 primer on the genomic sequence (data not shown). Moreover, primer extension analysis performed using a primer located in the second intron indicates the presence of several transcription initiation sites within this intron (data not shown).

The galectin-3 mRNA transcribed from the distal promoter were detected in a single round of 30 PCR cycles using MN-38 and a 5′ primer, GAL04, located within the 5′ untranslated part of the cDNA upstream of the isolated intron (Fig. 4). These results indicate that in HOS cells both mRNA species are produced. However, the relative amount of transcripts containing the intron sequences, detected only after two rounds of PCR, is scarcer than the mRNA transcribed from the distal promoter. This was confirmed by Northern analysis which shows a faint signal after hybridization with a probe specific for the intron sequence and long exposure time of the autoradiogram while the normal mRNA are easily detectable after hybridization with a cDNA probe (data not shown).

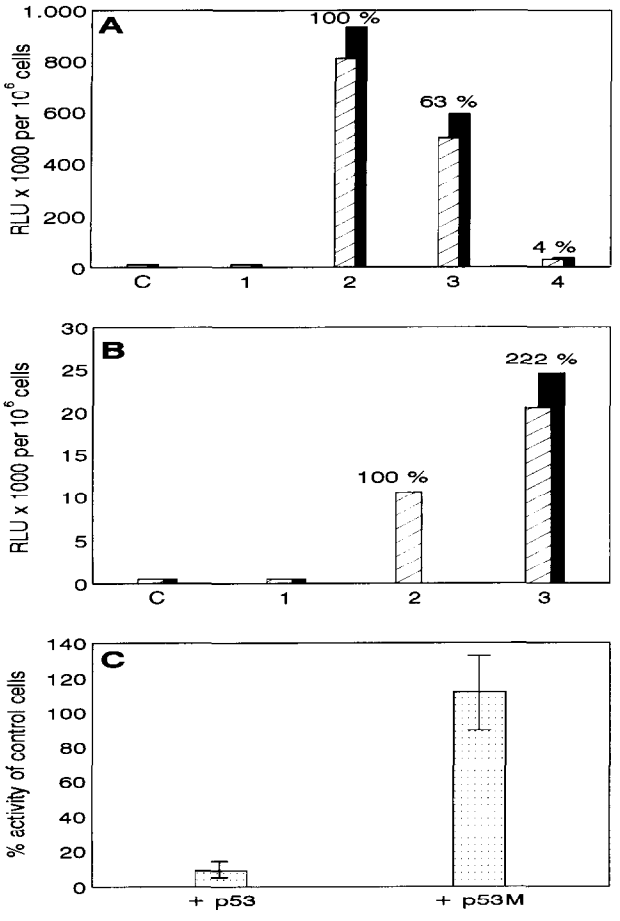

Fig. 3. Promoter activity of the cloned intron from the galectin-3 gene in the absence and in the presence of wild-type or mutated p53. (A,B) Transient transfections were conducted on 10^6 HOS cells (panel A) or J774 clone E cells (panel B) with a complex of 5 μg of plasmid DNA and 12.5 μg of lactosylated poly-L-lysine. Negative controls included mock-transfected (lanes C) and promoter-free plasmid pGEM-LUC transfected cells (lanes 1) while a positive control was obtained with pSV2-LUC transfected cells (lanes 2). Cells were transfected with the plasmid pGEM99-LUC which contains the intron sequence inserted upstream of the firefly luciferase gene reporter in direct orientation (lanes 3) or with pGEM99R-LUC in which the intron is in reverse orientation (lane 4). Luciferase activity is expressed in RLU (relative light units) per 10^6 cells and as a percentage of that obtained with pSV2-LUC transfected cells. Hatched and black columns represent independent experiments. (C) 10^6 HOS cells were co-transfected with 5 μg of pGEM99-Luc and 0.5 μg of pBR322 or with 0.5 μg of a plasmid encoding wild-type p53 (lane +p53) or mutated p53 (lane +p53M). Luciferase activity is expressed as a percentage of that obtained from control cells which were co-transfected with pGEM99-LUC and pBR322. These values are the results of eight independent experiments.

4. Discussion

In an attempt to understand the regulation of the expression of the human galectin-3 gene, we undertook to isolate the 5′ flanking sequence of the gene by genome walking using the LM-PCR technique. Because the 5′ untranslated region of the mRNA is not well defined due to variability of these sequences [5,29], we decided to initiate the genome walking a few base pairs downstream of the ATG codon. Upon sequencing, the amplified PCR product revealed the presence of a 651 bp intron located 18 nucleotides downstream of the ATG translation initiation codon, contained within a short exon of 22 bp. By homology with the mouse galectin-3 gene [29], this intron appears to be the second intron of the gene.

The sequence analysis of the PCR fragment revealed typical regulatory elements found in numerous eukaryotic genes transcribed by polymerase II [31]. This intron inserted upstream of a promoter-free luciferase gene acts as a strong promoter, leading to a high level of luciferase activity in human cells transfected with such a construct. The promoter activity of the second galectin-3 intron is shown to be as active as much as or even more so than the SV40 early region. Additional data indicate that this internal promoter activity is not specific for the human galectin-3 gene and that the rabbit gene also contains a strong internal promoter in the corresponding intron (data not shown). These results raise the question of the use of this internal promoter in the cells. Are the two promoters mutually exclusive or can they be used simultaneously, and do the cells have the ability to switch promoters? The detection of transcripts specific for the two promoters, by RT-PCR analysis (Fig. 4), may suggest that the activity of the two promoters are independent rather than exclusive. Transfection of HOS cells with a vector over-expressing the transcripts initiated from the internal promoter do not exhibit a specific modulation of the activity of the distal promoter (data not shown), suggesting that indeed the two promoters are independent.

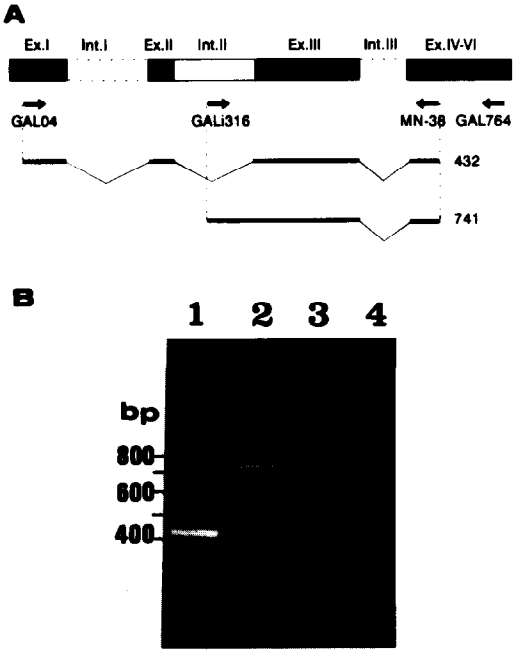

Fig. 4. Transcriptional activity of the internal promoter from the cloned intron in HOS cells. (A) mRNA was reverse transcribed from the primer GAL764. Two rounds of PCR were subsequently performed using the 3′ primer MN-38 upstream of GAL764 and with the 5′ primer GALi316 located in the intron sequence. Alternatively, PCR was initiated with the 5′ primer GAL04, 5′-GTCCGCAGCCAGCCAACGAG-3′, located from nt −26 to −7 in the 5′ part of the untranslated mRNA produced by the distal promoter. The numbers indicate the expected size of the amplified cDNA in bp. Exons (Ex.) and introns (Int.) are not drawn to scale. (B) PCR products were analyzed by gel electrophoresis. In lane 1, an expected 432 bp fragment is detected after PCR initiated with the primers GAL04 and MN-38. In lane 2 are products obtained after PCR initiated with the primers GALi316 and MN-38. In lanes 3 and 4 are samples amplified under the same conditions as in lanes 1 and 2 except that the reverse transcription step was omitted. The size marker is a 100 bp DNA ladder.

The expression of the gene is modulated upon replicative competence of cells [17], and is constitutively high in tumour cells, particularly in advanced stages of colorectal cancer [14]. This may suggest that the galectin-3 gene is controled by transcription factors altered during tumorigenesis. Because the p53 tumour-suppressor gene encodes a transcription factor which is frequently damaged in this type of cancer [32] and because the second galectin-3 intron contains a p53 binding site consensus sequence, we looked for a putative modulation of the promoter activity of the second galectin-3 intron by wild-type and mutated p53. Co-transfection of a plasmid encoding wild-type p53 produces a drastic decrease in promoter activity. This effect is specific of wild-type p53 and is not detected with a plasmid containing a point mutation in the p53 cDNA. This result demonstrates that p53 controls the activity of the internal promoter of galectin-3 gene as it does for others genes involved in the ongoing cell proliferation [33]. Experiments are in progress to determine if the distal promoter may be controled by the p53 status of the cells.

The determination of the transcription initiation site and the cloning of mRNA initiated from the internal promoter from the second intron of the galectin-3 gene in several human cells are under current investigation in order to define precisely the structure and the putative coding sequence of this mRNA. The data will be expected to give some insight on elements required to understand the appropriate regulation of the gene during the normal cell cycle as well as in case of pathological disorders.

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