Characterization and functional dissection of the galectin-1 gene promoter

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Abstract The galectin-1 gene encodes a β-galactoside-binding protein whose overexpression is associated with neoplastic transformation and loss of differentiation. Transient transfection assays of a series of deletions constructs (pGAT) showed that the galectin-1 promoter is highly active in cells both expressing and non-expressing the endogenous gene, and that the basal activity is determined by sequences encompassing the transcription start site (−50/+50). Both an upstream (−50/−26) and a downstream position-dependent (+10/+50) *cis*-elements are necessary for efficient transcriptional activity and are able to bind nuclear proteins.

Key words: β-Galactoside-binding protein; Eukaryotic cell; Promoter; Initiator

1. Introduction

Galectin-1 is a member of galectins, a family of structurally related proteins that share the ability to bind β-galactosides through a conserved carbohydrate recognition domain [1–3]. Galectin-1 has been implicated in such biological processes as cell–cell [4] and cell–matrix interaction [5], cell differentiation and growth regulation [6,7], maturation of T-lymphoblastoid cells [8] and in tumor progression [9]. Galectin-1 is a developmentally regulated gene [10] whose activity is strongly modulated during cell differentiation and transformation. Acquisition of tumorigenic phenotype and loss of differentiated functions are often accompanied by strong stimulation of galectin-1 expression in several cell systems and in human tumors [11–14].

Galectin-1 expression in rat thyroid and liver cell lines is very low or undetectable but increases 100-fold upon transformation of these cells by cellular or viral oncogenes [15]. By contrast, treatment with a differentiating agent of some transformed cells leads to extinction of galectin-1 expression [16,17]. Nuclear run-on experiments showed that such regulation of this gene occurs, at least in part, at the transcriptional level [15]. Finally, somatic cell hybrids obtained by fusing human osteosarcoma expressing cells with rat liver differentiated non-expressing cells, show a loss of both differentiated functions and tumorigenic phenotype, and activation of the previously silent rat galectin-1 alleles [18].

We have previously characterized the structure of the mouse galectin-1 gene and identified the transcription start site [19]. Because of the apparent significance of the galectin-1 gene during development and transformation, we have initiated studies to define functional elements of the galectin-1 promoter by transient transfection experiments of promoter constructs and by band-shifting experiments. We found that a small DNA region of no more than 100 bp surrounding the transcription start site (−50/+50) accounts for most of the transcriptional activity. This regulatory region includes two elements that are both required for efficient transcription in cultured cells and that can bind proteins present in nuclear extracts. One element is located just upstream from a putative TATA box; the other one is located between 10 and 50 bp downstream from the transcription initiation site and its function is position-dependent. Finally, we show that all the promoter constructs containing at least these two elements and up to 2300 bp upstream from the transcription start point, are highly active when transfected in cultured cells either expressing or not the endogenous gene.

2. Materials and methods

2.1. Plasmid construction

The galectin-1 promoter fragments were excised from the previously described pHS2 plasmid [19] by appropriate restriction endonucleases. An *Hin*dIII/*Xmn*I fragment, spanning the genomic region −2300/+50 relative to the transcription start site, was cloned in the pUC19 vector. The 5′ deletions were obtained by cleavage at unique sites with *Eco*RI, *Stu*I, *Pst*I, *Nar*I, and *Sma*I restriction endonucleases, respectively. Smaller promoter fragments contained in pGAT50, 26 and 16, respectively, were obtained by PCR amplification using appropriate oligonucleotides containing an artificial *Sma*I restriction site at the 5′-end. For the D series, a 40 bp *Ava*I–*Xmn*I fragment of the first exon (+10 + 50) was deleted. The same deleted fragment was cloned in tandem and in both directions in the pGAT50D vector to obtain the F40 plasmid series. The fragments were excised from the vector using appropriate restriction enzymes, flush-ended at the 5′ end by Klenow polymerase when required, and then cloned in the *Sma*I site upstream from the CAT reporter gene in the pEMBL8CAT expression vector. All constructs were checked by sequencing with the chain-termination method [20]. Chimeric pGAT plasmids were purified with the Plasmid Maxi Kit (Qiagen).

2.2. Cell culture, transient transfections and CAT assays

NIH3T3 mouse fibroblast, PC Cl3, PC *myc/raf* rat thyroid cells [15] and FAO rat liver cells [21], were cultured in DMEM supplemented with 10% foetal calf serum (IGNI Flow), plated at a density of about 250,000 cells per 60 mm Petri dish 16 h before transfection. DNA transfections were carried out by the calcium phosphate precipitation method [22]. Cultures were co-transfected with 10 μg of test plasmid and 2 μg of a vector carrying the luciferase or β-galactosidase genes under the control of the CMV and SV40 promoter element, respectively (pCMV-LUC, pSVβ-gal) as internal standard for transfection efficiency. 2.5 h after transfection, PC Cl3 and PC *myc/raf* were exposed to 15% glycerol in HEPES-buffered saline solution for 1 min and then re-fed with growth medium. Cell extracts were prepared 48 h after tranfection by several cycles of freezing and thawing. CAT assays were performed as described [22]. Protein concentration was determined by the Bio-Rad assay. The CAT activity in the extract of the transfected cells was quantified by counting the amount of [^{14}C]chloramphenicol converted to the mono-acetylated forms, normalized to the luciferase or to β-galactosidase activity. Luciferase activity in cell extracts was

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measured essentially according to de Wet et al. [23]. Cells were rinsed once with phosphate buffer and then lysed in 0.2 M Tris (pH 7.6) + 0.1% Triton X-100 (0.2 ml for 60 mm dishes). Lysed cells were scraped and spun for 2 min. Supernatants were recovered for luciferase and protein assays. Luciferase activity was determined using the luminometer Monolight 2010 (Analytical Luminescence Lab). 20 μl of lysates were automatically mixed with 100 μl of the luciferase substrate solution (20 mM Tris, pH 8, 4 mM $MgSO_4$, 0.1 M EDTA, 30 mM DTT, 0.5 mM ATP, 0.5 mM D-luciferin, and 0.25 mM coenzyme A) and emitted fluorescence was measured for 15 s. 40 μg of total protein extract were assayed for β-galactosidase activity as previously described [22]. Equal amounts of β-galactosidase activity (routinely 1 pM corresponding to about 100 μg of total protein extract) were used for the determination of CAT activity. Each transfection experiment was repeated at least three times and gave reproducible results.

2.3. Nuclear extracts and gel-retardation assays

Nuclear protein extracts were prepared as described [24]. Fragment A (−60/+5), fragment B (−50/+5) and fragment C (+10/+50) were used as probes for binding reactions. Labelling reaction was performed using Klenow polymerase (Amersham) and [α-^{32}P]dATP or [α-^{32}P]dGTP or T7 polynucleotidekinase (Boehringer-Mannheim) and [γ-^{32}P]dATP. Synthetic double-stranded oligonucleotides used as competitors were purchased from Promega. Gel-shift assays were performed as described [25], in the presence of 3 μg poly(dI/dC) per sample as non- specific competitor. Samples were loaded on 5% non-denaturing polyacrylamide gels. The gels were then dried and subjected to autoradiography. For gel retardation competition assays, various amounts of cold competitor DNA were added during preincubation.

3. Results

3.1. Deletion analysis of galectin-1 promoter region

To localize *cis*-elements that regulate the efficiency of the galectin-1 gene promoter, a series of 5′-deleted promoter fragments, maintaining 50 bp 3′ from the initiation site, were fused to CAT gene in the pEMBL8CAT vector and used for transient transfections into the NIH3T3 mouse fibroblast cell line. This cell line expresses high levels of endogenous galectin-1 mRNA (data not shown). Plasmids pEMBL8CAT and pRSV-CAT (containing the Rous sarcoma virus long terminal repeat) were used as negative and positive control, respectively. The fusion constructs with their relative CAT activity are shown in Fig. 1. Construct pGAT-2300, containing 2300 bp upstream from the initiation start site, is able to direct the transcription of the reporter gene with an efficiency comparable to that of the pRSV-CAT positive control. Sequences up to 60 bp upstream from the transcription start site could be removed without any significant loss of CAT activity. Removal of sequences containing two overlapping Sp1 binding sites reduced the promoter activity to 65% (plasmid pGAT-50). Deletion of further sequences to −26 almost completely abolished CAT activity (plasmid pGAT-26). The 5′ deletion analysis suggests that galectin-1 promoter sequences from −50 to +50 are sufficient to drive efficient expression of a reporter gene, and that nucleotides between −50 and −26 are essential for promoter activity. The presence of the Sp1 consensus sequences (−60/−50) slightly increases the transcription efficiency but is not a major determinant of the basal activity.

Table 1
Galectin-1 promoter activity in different cell lines

Plasmid	Relative CAT activity*			
	NIH3T3	FAO	PC C13	PC *myc/raf*
pGAT2300	100 ± 9.2	90 ± 7.5	110 ± 8.8	100 ± 6
pGAT60	95 ± 2	100 ± 7.8	90 ± 9.2	110 ± 8.2
pGAT50	65 ± 7.5	60 ± 5.1	70 ± 4.3	75 ± 5
pGAT26	10 ± 3.3	5 ± 1	5 ± 1.1	15 ± 3.2

*Expressed as a percentage of activity obtained by transfection of pRSVCAT in NIH3T3 which was arbitrarily assigned 100% and normalized for luciferase activity of the extract.

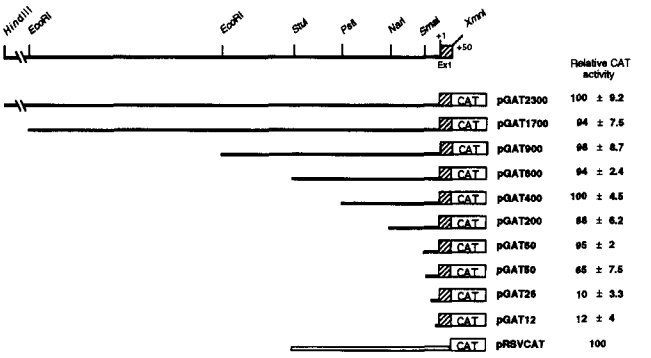

Fig. 1. Deletion analysis of the galectin-1 gene promoter region. (Top) Schematic diagram of the mouse galectin-1 gene 5′-flanking region. The first exon (dashed box), the start site of transcription (+1) and some restriction sites are indicated. (Bottom) 5′-deleted clones contain 50 bp of the first exon. All the constructs shown were transiently transfected in NIH3T3 cells. Relative CAT activity was normalized to that of the pRSVCAT, which was arbitrarily assigned 100% activity. All extracts were adjusted to identical luciferase or β-galactosidase activity. The results shown represent the averages of at least five independent transfection experiments with two plasmid preparations. Standard deviations of the means are indicated.

3.2. Analysis of the downstream region

To determine whether there are regulatory sequences downstream the start site, further constructs maintaining only 9 bp 3′ from the transcription start were prepared. With these deletion mutants, pGAT2300Δ (−2300/+9), pGAT60Δ (−60/+9) and pGAT50Δ (−50/+9), the CAT activity was almost completely abolished (Fig. 2). This result indicates that downstream sequences, located between +10 and +50, are essential for promoter activity. Comparing the results obtained with plasmids pGAT50Δ and pGAT26, it is clear that the downstream +10/+50 and the upstream −50/−26 elements are interdependent and both necessary for efficient transcription. We next generated further constructs in which the downstream sequence +10/+50 was inserted, in both directions and in tandem, upstream from the −50/+9 region (plasmids pGAT-F40, pGAT-F40inv and pGAT-2F40inv). Transfection of these plasmids revealed that the CAT activity was not restored (Fig. 2). These results suggest that the function of the downstream element is position-dependent.

3.3. Nuclear proteins bind both upstream and downstream elements

To investigate if nuclear factors bind sequences located around the transcription start site, we performed mobility shift experiments using as probes the three DNA fragments A, B and C (Fig. 3). Fragment A corresponds to the −60/+5 region and includes two overlapping Sp1 consensus sequences; the results (Fig. 4A) showed that a complex is formed with NIH3T3 nuclear extract that is specifically inhibited by the addition of a 50-fold molar excess of both cold fragment A and a cold dou-

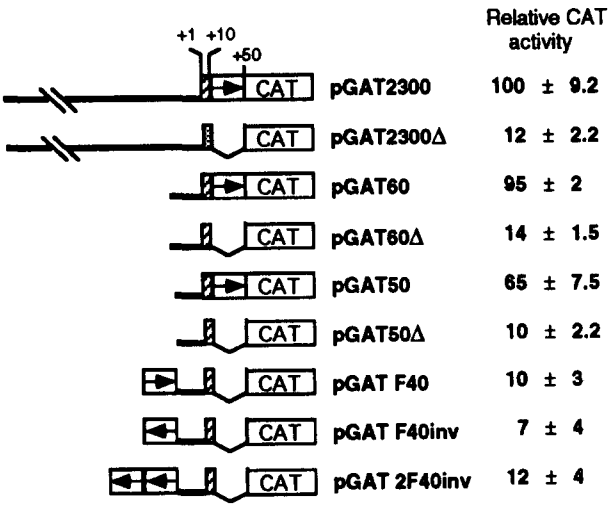

Fig. 2. Effects of deletion or translocation of the +10/+50 region on the activity of pGAT constructs. The *AvaI/XmnI* (+10/+50) fragment was deleted from the pGAT-2300, pGAT-50 and pGAT26 plasmids (pGAT*Δ*), or translocated upstream from the start site in both orientations and in tandem (pF40 series). The +1/+10 region (hatched box) and the +10/+50 (open box with arrow indicating the 5′–3′ orientation) are indicated, as are the deleted sequences. The CAT activities shown on the right are expressed relative to that of pRSVCAT, which was arbitrarily assigned 100%. The results represent the averages of at least five independent transfection experiments. Standard deviations of the means are indicated.

ble-stranded oligonucleotide containing a consensus sequence for the binding of Sp1 factor. This experiment indicated that Sp1 transcription factor binds this fragment.

To investigate if less abundant factors are able to bind these sequences we used fragment B as a probe. Fragment B (−50/+5) consists of the same region except for the Sp1 consensus sequences that have been deleted; the results of this experiment (Fig. 4B) indicated that complexes are formed and specifically inhibited by the addition of a 50-fold molar excess of cold fragment B but not by an oligonucleotide containing Sp1 consensus sequences. This suggests that nuclear factors other than Sp1 bind specifically to sequence −50/+5.

The last probe used in these experiments was the *AvaI/XmnI* fragment C that corresponds to the downstream element described above (+10/+50). Also in this case retarded bands were specifically competed by a 50-fold molar excess of the cold fragment C but formation of the complexes was not inhibited by an unrelated competitor double stranded oligonucleotide (Fig. 4C). We next verified whether known transcription factors were involved in the complex with fragment B or fragment C. We performed competition assays with a 100-fold molar excess of several cold commercially available double-stranded oligonucleotides (Promega Corp., Madison, WI). These included oligonucleotides containing DNA binding sites for the following transcription factors: AP1, AP2, OCT1, CTF/NFI, GRE, AP3, CREB and NFkB. In no case was the retardation of both fragment B or fragment C eliminated (data not shown). These results indicate that both downstream and upstream elements, which have been shown to be necessary and sufficient to drive efficient expression, are bound by yet unidentified nuclear proteins.

3.4. Tranfections of gal/CAT constructs in galectin-1 expressing and non-expressing cell lines

To identify possible *cis*-elements of the galectin-1 gene regulatory region involved in the differential expression of the endogenous gene in different cell lines, we performed transfection experiments of some representative pGAT constructs (pGAT-2300, −60, −50 and −26) in PC Cl3, PC *myc/raf* and FAO cell lines. PC Cl 3 is a rat thyroid differentiated cell line in which the galectin-1 mRNA levels are very low, while PC *myc/raf* is the transformed high tumorigenic counterpart that shows a 100-fold increase of galectin-1 expression [15]; FAO are rat liver differentiated cells in which the galectin-1 mRNA is not detectable by Northern blot assay [18]. The results of the CAT assays are summarized in Table 1. In all cell lines the CAT activity was comparable for each transfected plasmid with that observed in NIH3T3 extracts. This result indicates that sequences responsible for the differential expression of the endogenous gene are not present or not functional in the genomic region −2300/+50 of the galectin-1 gene.

4. Discussion

Galectin-1 gene expression is developmentally regulated and may vary dramatically during tranformation events both in cell lines and in mammalian tissues. For this reason in the present work we have focused our attention on the identification of the DNA sequences necessary for the basal transcription of the galectin-1 gene and of possible *cis*-elements involved in the differential expression of the gene. To our knowledge this is the first characterization of the regulatory region of a gene coding for a galectin.

4.1. Identification of minimal promoter elements

Using transient CAT assay we have demonstrated that the galectin-1 minimal promoter is composed of sequences from −50 to +50 relative to the transcriptional start site; the presence of two overlapping Sp1 binding sites located between −50 and −60 moderately increases the transcriptional efficiency. This means that transcriptional activators other than Sp1 are responsible for basal promoter activity. Moreover, the expression of CAT gene was for each pGAT plasmid comparable in strength to that produced by SV2CAT and RSVCAT in which strong viral promoters drive the CAT gene. We also show that the galectin-1 minimal promoter is regulated by the interaction of at least two elements: the first is located between −50 and −26, just upstream from a sequence that resembles a

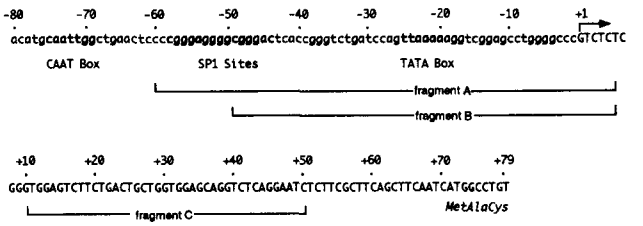

Fig. 3. Nucleotide sequence of the mouse galectin-1 gene region surrounding the transcription start site. Putative regulatory elements (CAAT and TATA box and Sp1 binding sites) are in bold. The arrow indicates the transcription start site (+1). Capital letters indicate the transcribed sequences. The first three amino acid of the first exon are indicated. The fragments used for the band-shifting experiments are shown (fragments A, B and C).

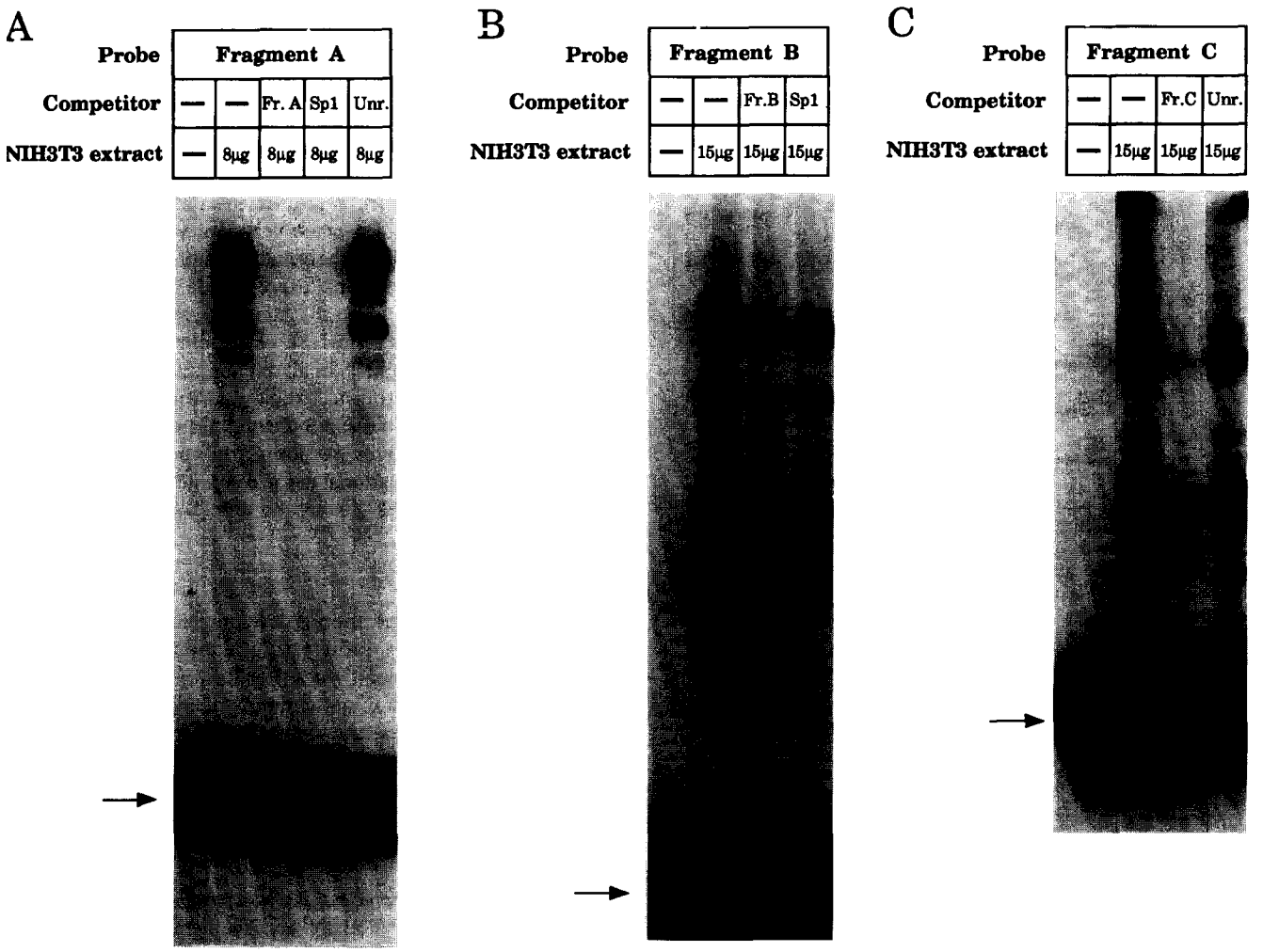

Fig. 4. Gel electrophoresis mobility shift analysis of the galectin-1 gene −60/+50 region. (A) Fragment A (−60/+5) was used as a probe. The specific complex was eliminated by competition with a 50-fold molar excess of cold fragment A or of cold double-stranded oligonucleotide containing an Sp1 consensus sequence (ATTCGATCGGGGCGGGGCGAG). No competition was observed in the presence of a 200-fold molar excess of an unrelated DNA fragment (TCGGAAGTCGTAGCCTGAGGTTCGTAGCTT CG). (B) Fragment B (−50/+5) was end-labelled and the specific complex was competed by a 50-fold molar excess of unlabelled fragment but not by a 200-fold molar excess of the oligonucleotide containing an Sp1 binding site. (C) The +10/+50 fragment C was used as a probe. The complex was inhibited by a 50-fold molar excess of fragment C and not by a 200-fold molar excess of the unrelated DNA fragment. Arrows indicate the unbound probes. Exposure time for panel A was 12 h and for panel B and C was 72 h.

TATA box; the second is located downstream from the transcription start (+10/+50). When either element is removed, the transcription efficiency drops dramatically. A similar structure has been found for the glial fibrillary acidic protein promoter [26].

The analysis of the upstream element does not reveal any canonical sequence for the binding of known transcription factors; the presence of −50/−26 sequences could be necessary for the binding of regulatory factors to the putative adjacent TATA box. This could explain why no efficient transcription is driven by the pGAT-26 plasmid that contains the putative TATA box but no other nucleotide at its 5′-end. Alternatively, the TATA sequence could be non-functional and other *cis*-elements could exert its function. Interestingly, the region surrounding the transcriptional start site (−3/+7) of both the murine (CCCGTCTCTC) and the human galectin-1 gene [27] (CCCATCTCTC), matches the consensus sequence for initiator elements (Inr) (−3 YYCAYYYYYY +7) [28] and shows homologies with the Inr regions of other genes [29]. Inr elements have been described both in the context of TATA box-containing and in TATA-less promoters [30] and appear to be essential for accurate transcription initiation. The Inr elements can function cooperatively with other transcriptional elements including TATA boxes or their counterparts, and with downstream regulatory regions, as in the case of the terminal deoxynucleotidyltransferase (TdT) promoter [30].

The downstream region (+10/+50) is also required for maximal basal transcription and its function appears to be strictly position-dependent because constructs with this fragment positioned upstream of the start site are not active (Fig. 2). The possibility must be considered that the deletion +10/+50 interrupted a functional element, and the apparent position dependence in fact reflects only that the whole element was not transplanted. However, the band shifts observed with fragment C argue that some complete element is indeed present in this region. The +1/+30 region is perfectly conserved among differ-

ent species, while the adjacent sequences are only partially conserved. This also points to the possible crucial role of this region in the regulation of galectin-1 gene expression.

4.2. Activity of galectin-1 promoter in different cell lines

The modulation of galectin-1 expression during development, the striking increase of galectin-1 mRNA levels in transformed cells and in tumors and the observation that this regulation occurs at the transcriptional level [14,15], prompted us to study the activity of the promoter constructs in differentiated and in transformed cell lines. The cell lines used in this study showed differences in endogenous galectin-1 mRNA levels of up to 100-fold. The data described here indicate that in transient transfections, the expression of various galectin-1-promoter/CAT vectors, containing at least the minimal promoter and up to 2300 bp of the upstream region, was comparable in strength among the different cell lines. These results indicate that the differential expression of the endogenous galectin-1 gene cannot be reproduced by transient transfection of the constructs used in this study.

We previously demonstrated that cell hybrids resulting from the fusion of human osteosarcoma (expressing galectin-1 gene) with rat FAO cells (non-expressing) show activation of the FAO galectin-1 alleles [18]. These data suggested that the donor cells contain regulatory factors that activate FAO galectin-1 alleles. Now we show that promoter constructs are transactivated by factors present in both expressing and non-expressing cells. Thus, we can hypothesize the existence of a repressor that acts *in trans* on a genomic region not contained in our constructs, and a mechanism of derepression that activates the gene in expressing-cells and in hybrids. Alternatively, the gene could be repressed in non-producing cells through a mechanism in *'cis'*, non-functional on transiently transfected promoters, and derepressed in the hybrids in a dominant fashion. In this respect it is noteworthy that the treatment of FAO cells with 5'-azacytidine, a DNA demethylating agent, leads to activation of the galectin-1 gene [18]; these observations suggest that DNA methylation is involved in the regulation of galectin-1 gene expression in different cell lines.

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