

Cloning and characterization of *Echinococcus granulosus* (Cestode) *EgactI* and *EgactII* actin gene promoters and their functional analysis in the NIH3T3 mouse cell line

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Abstract

We report here for the first time the structure and function of a promoter from a cestode. The ability of DNA fragments respectively encompassing the 935-bp and 524-bp regions upstream from the ATG codon from the *EgactI* and *EgactII* actin genes of *Echinococcus granulosus* to promote transcription was studied in the NIH3T3 mouse cell line. The results of transfection assays showed that both regions have strong promoter activity in these cells. The fragments were tested in both orientations and the 524-bp fragment of *EgactII* presented a bidirectional promoter activity. Deletion analysis of *EgactI* and *EgactII* promoters indicated the presence of regulatory regions containing putative silencer elements. These results indicate that both *EgactI* and *EgactII* promoters are functional and that the preliminary functional evaluation of *E. granulosus* and possibly of other cestode promoters can be performed in heterologous cell lines.

Key words

- *Echinococcus granulosus*
- Actin
- Promoters
- Cestode
- Gene expression
- Deletions

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Introduction

Echinococcus granulosus (Cestode) is the causative agent of hydatidosis, which is a zoonosis affecting human and animal health all over the world (1). The South of South America includes some of the countries with the most prevalent levels of infections with this parasite. There is a special interest in the study of the molecular mechanisms involved in the development and differentiation processes during the life cycle of *E. granulosus*, particularly concerning the unusual ability of protoscolices to differentiate into either

adult worms or hydatid cysts, depending on environmental conditions.

Actin genes and their transcriptional controlling elements are certainly important targets of such differentiation processes, since actin-coordinated cell movements are essential in cell growth and differentiation. Additionally, the events that determine which member of the actin gene family will be expressed in a given tissue and at a given moment also involve the interaction of regulatory proteins with specific sequences located within or immediately flanking the gene of interest (2).

Based on the conservation of actin genes in eukaryotes and on the temporal/spatial regulation of expression of many members of this multigene family in different organisms (3), our laboratory has used these genes as a model to study the basic mechanisms of transcription regulation in this parasitic flatworm. We have focused our attention on the *cis*-linked regulatory regions required for transcriptional activity of two actin genes from *E. granulosus*, namely *EgactI* and *EgactII*, previously described by our group (4). Functional assays for *E. granulosus* and other cestode promoter sequences are problematic since homologous cell lines are not available, and techniques to efficiently transform these organisms have not yet been developed. In spite of these limitations, the activity of promoters from *Schistosoma mansoni* (Trematode), *Artemia franciscana* (Crustacea) and *Ephydatia muelleri* (Porifera) genes has been already analyzed with success using mammalian cell lines, suggesting conservation of the general machinery of transcription (5-8). Based on this conservation of the basic transcription apparatus, we also decided to test the activity of *E. granulosus* actin gene promoters on the NIH3T3 mammalian cell line. The present study provides the first report of the structure and function of a promoter from a cestode. We describe the promoter analysis of the *EgactI* and *EgactII* actin genes, showing their putative regulatory regions and function in driving chloramphenicol-acetyltransferase (CAT)-reporter gene transcription in the NIH3T3 mouse cell line.

Material and Methods

Plasmids and DNA manipulation

All DNA manipulations were carried out by standard techniques and plasmid structures were confirmed by restriction digestion (9). Sequencing was performed by dideoxynucleotide method using the T7 Se-

quencing™ Kit (Pharmacia, Uppsala, Sweden) or by cycle sequencing (DNA Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit, Amersham, Little Chalfont, England). Nucleotide sequence analysis was performed using SIGNAL SCAN (10), MatInspector (11) and GCG computer programs (12). Vectors pCAT Basic and pCAT Enhancer were purchased from Promega Corp. (Madison, WI, USA). Plasmid pCMVβgal was a gift from Dr. Vilma Martins (Ludwig Institute for Cancer Research, São Paulo, SP, Brazil). Supercoiled plasmid DNAs were purified using Qiagen tips.

Plasmid constructs

Actin gene promoter-CAT reporter constructs containing 5'-flanking regions (Figure 2) were prepared by PCR amplification using as templates genomic DNA fragments encompassing the entire *EgactI* and *EgactII* actin genes (4). The 5'-flanking regions from -935 to -14 and -524 to +50 from *EgactI* and *EgactII*, respectively, were cloned into the promoter-less pCAT Basic vector, generating the constructs pEgI-935CAT and pEgII-524CAT or into the pCAT Enhancer vector, generating the plasmids pEgI-935CATE and pEgII-524CATE. We also constructed plasmids in which these same sequences were cloned into pCAT Basic in the reverse direction (plasmids pEgI-935CATR and pEgII-524CATR). The primers used to amplify the *EgactI* promoter region from -935 to -14 were: -935/-917 (5'-AAAAAAGCTTCAA AATTTAACACACGAAA-3') and -31/-14 (5'-AAAAGTCGACATTTGTCTTCTC TACTGC-3') and the primers used to amplify the *EgactII* promoter region from -524 to +50 were: -524/-507 (5'-AAA AAAGCTT CTCATATTGAAGAAGGCA-3') and +34/+50 (5'-AAAAGTCGACATACCAGAGC CGTTGTC-3'). The oligonucleotides used as 5' PCR and 3' PCR primers each contained a *HindIII* and a *SallI* restriction enzyme site (underlined), respectively. Oppo-

site sites were designed in the oligonucleotides used to amplify the fragments that were cloned in the reverse direction.

pCAT Basic plasmids containing deletions of both 5'-flanking regions encompassing the 935- and 524-bp 5' regulatory regions were also constructed (Figure 2). Plasmids pEgI-717CAT and pEgI-525CAT were prepared with the -717/-700 (5'-AAAA AAGCTTGTTTACATAAGGGAAGTC-3') or -525/-509 (5'-AAAAAAGCTTTCATTC ACAAGAGGGAT-3') 5' primers and with the common *EgactII* 3' oligonucleotide primer -31/-14. Both the pEgI-223CAT and pEgI-100CAT constructs were derived from the pEgI-525CAT plasmid. Plasmid pEgI-525CAT was hydrolyzed with both *HindIII* (at -525) and *SacI* (at -223) or with both *HindIII* (at -525) and *PstI* (at -100), blunted and ligated, generating the constructs pEgI-223CAT and pEgI-100CAT, respectively. Plasmids pEgII-387CAT, pEgII-288CAT and pEgII-128CAT were prepared with the -387/-365 (5'-AAAAAAGCTTTATATGATATC AGGACAGCCCTCT-3'), -288/-271 (5'-AAAAAGCTTAAGACGTAAAGCATTATT-3') or -128/-111 (5'-AAAAAAGCTTCCCC TTCGATGAGGTTAA-3') primers and with the common *EgactII* 3' oligonucleotide primer +34/+50. The 5' and 3' PCR primers contained *HindIII* and *Sall* restriction sites (underlined) at their 5' ends. All PCR reactions were performed in 1X ThermoPol reaction buffer (New England Biolabs Inc., Beverly, MA, USA) with 200 mM of each nucleotide, 40 pmol of each primer, and 2 units of Deep Vent polymerase (New England Biolabs) with proof-reading exonuclease activity. The PCR fragments were digested with *HindIII* and *Sall* and ligated into the corresponding sites of pCAT Basic. Each construct was verified by sequencing at the 5' and 3' end point-plasmid junctions.

Cell culture conditions, transfections and CAT assays

NIH3T3 cells (obtained from Dr. Vilma

Martins) were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS) supplemented with 3.7 g/l sodium bicarbonate. For transient transfections, 1.9×10^5 cells were plated onto 35-mm dishes and grown to approximately 75% confluence before adding the transfection mixture. Eight microliters of Lipofectamin™ reagent (Life Technologies Inc., Rockville, MD, USA) was added to a mixture of 1 µg of CAT reporter gene containing plasmids and 1 µg of internal control plasmid pCMVβgal. This mixture was incubated at room temperature for 45 min in 200 µl of serum-free medium. Cells were washed once with 2 ml of serum free-medium and the transfection mixture was added. Cells were incubated for 5 h at 37°C in a CO₂ incubator. Following incubation, 1 ml of DMEM/20% FBS was added. The medium was replaced 18 h after the start of transfection. Forty-eight hours after transfection, cells were collected by centrifugation and washed once with phosphate-buffered saline, and lysates were prepared by freezing and thawing cells 4 times following resuspension in lysate buffer (0.25 M Tris, pH 7.8). Cell lysates were centrifuged for 15 min at 13,000 rpm at 4°C. Subsequently, the supernatant was assayed for β-galactosidase and CAT activities as previously described (9,13). CAT activities were normalized taking into account the β-galactosidase activities measured in all experiments. After chromatography, dried plates were autoradiographed using Hyperfilm™ ECL™ X and intensifying screens (DuPont, Boston, MA, USA). For quantification of acetylated ¹⁴C-labeled chloramphenicol, bands were cut out and submitted to scintillation counting. Promoter efficiency was expressed as the relative CAT activity. The means and standard deviations of CAT activity were calculated for 2 or 4 experiments and analyzed by the Student *t*-test. A probability of less than 5% ($P < 0.05$) was considered statistically significant.

Analysis of DNA-protein interaction by gel shift assay

Gel retardation assays were performed as previously described (13), with slight modifications: 5 µg of NIH3T3 total nuclear extract or bacterial lysate containing the human YY1 recombinant protein was incubated with 1 µg of poly (dI-dC) and 0.5 ng of 5' [³²P]-labeled DNA fragment (13) was then added. After 20 min at room temperature, protein-DNA complexes were separated from free probes on a 6% polyacrylamide gel in 0.5X TBE. The gels were dried and exposed to Kodak X-ray films. NIH3T3 nuclear extracts were prepared from 0.5 x 10⁶ to 1 x 10⁶ cells. Cells were washed with Tris-buffered saline (TBS), scraped from the plate with a rubber spatula, and centrifuged at 1,500 g for 5 min. The pellet was resuspended in 1 ml of TBS and transferred to another tube. Cells were again sedimented and the pellet was resuspended in 400 µl of ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF) and incubated for 15 min on ice. Twenty five microliters of 10% Nonidet P-40 was added and the mixture was submitted to vigorous vortexing for 10 s. Nuclei were sedimented for 30 s, resuspended in 50 µl of ice-cold buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF), and shaken for 15 min at 4°C. The nuclear extract was centrifuged for 5 min at 4°C and the supernatant containing the nuclear extract was stored at -70°C. All procedures were carried out at 4°C and protein concentration in the extracts was determined by the method of Bradford (14).

Plasmid pGEX-2T containing the human YY1-coding sequence was kindly provided by Dr. Thomas Shenk (Princeton University). To prepare bacterial lysate containing the GST-YY1 fusion protein we used a protocol described elsewhere (15), with slight modifications. The fusion plasmid was in-

roduced into bacterial strain JM101. Usually a liter of bacterial culture was grown to absorbance at 600 nm of 0.6. Synthesis of the fusion protein was induced with 1 mM IPTG. Bacteria were allowed to grow in the presence of IPTG for 4 h at 37°C and pelleted by centrifugation and the sediment was then resuspended in 10 ml of NETN (100 mM NaCl, 20 mM Tris, pH 8.0, 1 mM EDTA, and 0.5% NP-40). The suspension was sonicated 2-3 times for 1 min. All the steps were carried out at 4°C. The insoluble debris was removed by centrifugation at 17,300 g in a Sorvall SS34 rotor for 10 min. The supernatant containing the fusion protein was collected and stored at -70°C with 10% glycerol.

The DNA fragments containing specific promoter regions used in the gel retardation assays were obtained by PCR amplification by *Taq* DNA polymerase (CenBiot, Porto Alegre, RS, Brazil) using as templates genomic sequences containing *EgactI* and *EgactII* promoter regions (4). *EgactI* promoter regions from -935 to -718, -717 to -528, -525 to -224 and -223 to -14 were prepared as follows: the -935 to -718 fragment was amplified using the PCR primers -935/-917 and -737/-718 (5'-GAATCACCC GATCTTCTGGC-3'); the -717 to -528 fragment was amplified using the primers -717/-700 and -546/-528 (5'-GTATTACACAGGA AATTATT-3'); the -525 to -224 and -223 to -14 fragments were obtained by restriction enzyme digestion of the -525 to -14 PCR fragment with *XhoI* (site at -223). *EgactII* promoter regions from -524 to -389, -387 to -292, -288 to -131 and -128 to +50 were prepared using the following PCR primers, respectively: -524/-507 and -408/-389 (5'-TGGCCTGACCTCTGAAGGGT-3'); -387/-365 and -311/-292 (5'-TTGTAAAGCAG TTTTTTCA-3'); -288/-271 and -150/-131 (5'-GCAAACCTACCTGATGACTAG-3') and finally -128/-111 (5'-CCCCTTCGA TGAGGTAA-3') and +34/+50. All PCR and restriction enzyme digestion products

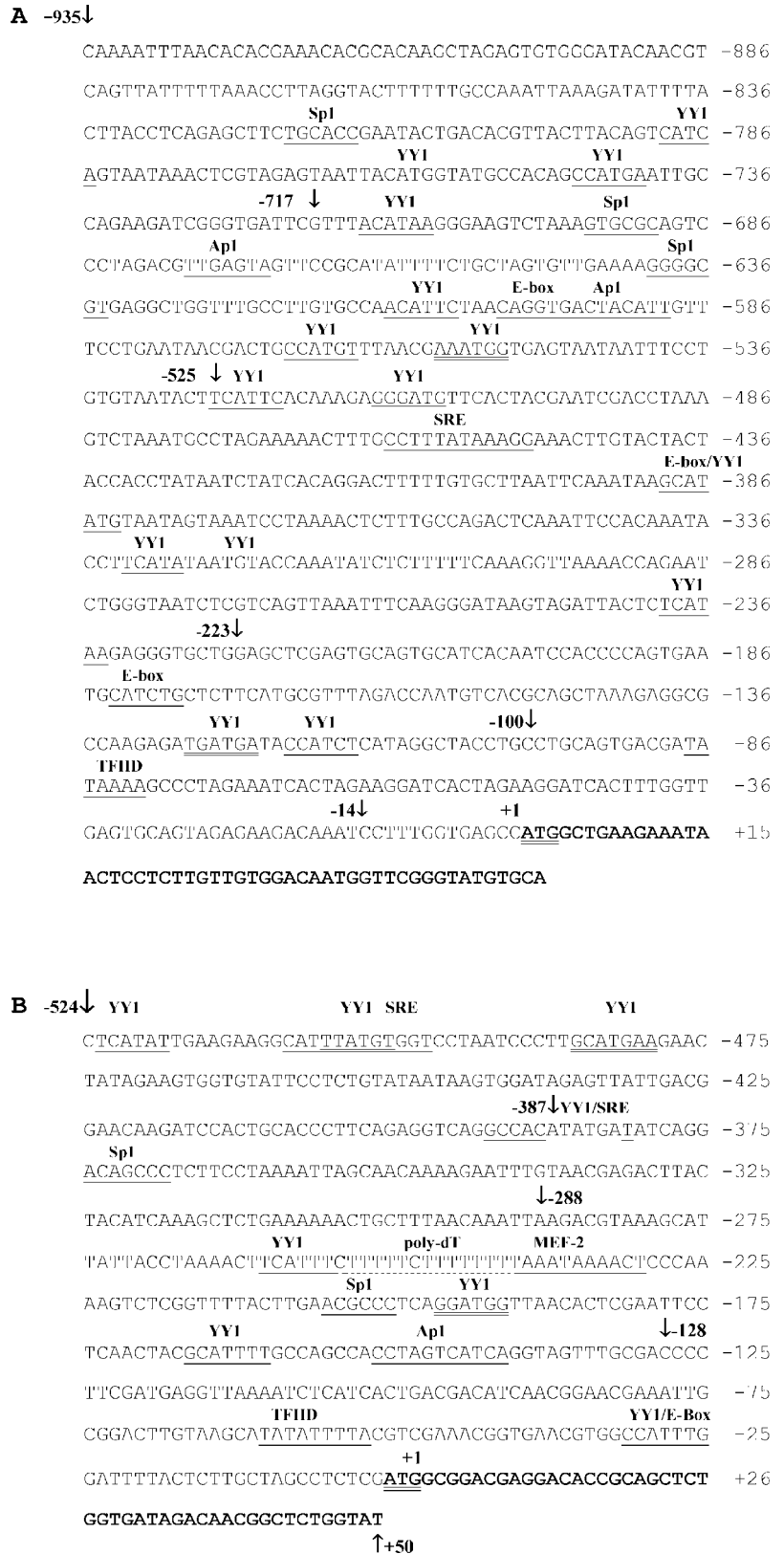
obtained were purified on polyacrylamide gels. An oligonucleotide containing the YY1 consensus binding site (5'-CGCTCCGCGG CCATCTTGGCGGCTGGT-3') was used as a positive control for YY1 binding.

Results and Discussion

Figure 1 presents the 5'-flanking sequences of the *EgactI* and *EgactII* actin genes. Nucleotide sequences were determined for the 935-bp and 524-bp regions upstream from the ATG (translation start codon), respectively (Figure 1A and B). These sequences did not present significant homology with each other or with other known promoters whose sequences have been deposited in the GCG data banks (12).

Computational sequence analysis of the 5'-flanking regions of *EgactI* and *EgactII* using SIGNAL SCAN (10) and MatInspector (11) revealed the presence of many binding sites for ubiquitous, developmental, cell type-specific transcription factors and putative elements that are known to contribute to the transcriptional regulation of actin genes. As indicated in Figure 1, E-box (16), SRE (17), and binding sites Sp1 (18), Ap1 (19) MEF-2 (20) and YY1 (21) were identified in both 5'-flanking sequences. However, these sequences are positioned differentially with respect to their ATG. The *EgactI* gene presents a canonical TATA box at nucleotide -87, while the *EgactII* gene promoter shows a TATA box-like sequence at nucleotide

Figure 1 - Nucleotide sequence of the *E. granulosus* *EgactI* (A) and *EgactII* (B) 5'-flanking regions. Nucleotides are numbered on the right in relation to the translation start codon (ATG), designated as +1 for the A of the Met initiation codon. Putative transcription factor binding sites are underlined. Putative binding sites for YY1 in the complementary strand are double underlined. The poly (dT) tract is underlined with a broken line. The coding sequence is shown in bold face type and the ATG translation start codon is double underlined. Arrows above the sequence denote 5' deletion end points and arrows below the sequence denote 3' end points.



-60. The *EgactII* 5'-flanking region also presents a poly (dT) element at nucleotide -257. Since most of these putative regulatory elements have been described to be functionally involved in the control of actin gene regulation, we suggest their putative role in the transcriptional control of *EgactI* and *EgactII* actin genes. Their different structural organization could also suggest a specific pattern of regulation. In spite of the presence of these putative regulatory elements, their role in the transcriptional control of these genes is unknown.

In order to study the functionality of these putative 5' regulatory regions in the transcriptional control of the *EgactI* and *EgactII*

genes, we prepared a series of actin gene promoter-CAT reporter gene constructs (Figure 2). The results of the functional assays are presented in Figure 2A and B. Both pEgI-935CAT and pEgII-524CAT determined a ~4-fold increase in CAT activity relative to pCAT Basic vector activity. These results showed that both 5' regions functioned as active promoters in NIH3T3 cells, suggesting that conserved transcription factors present in these mammalian cells recognize and bind the promoter regions promoting CAT transcription. Their promoter activities are relatively strong, since the levels of CAT expression were similar to those promoted by the SV40 promoter-CAT construct used as a positive control for these experiments (data not shown).

When tested in the presence of the SV40 enhancer sequence, the *EgactII* promoter activity increased approximately 5-fold, while the activity of the *EgactI* promoter decreased 1.2-fold when cloned in the same vector (compare pEgII-524CATE and pEgI-935CATE in Figure 2B and A). It has been reported that simultaneous Sp1 and Ap1 binding to the SV40 enhancer sequence and promoter elements can have significant effects on gene transcription (22,23). The activation or repression of *EgactI* and *EgactII* promoter activity could be the result of interactions between Sp1 and/or Ap1 NIH3T3 transcription factors bound to the promoter regions containing the respective binding sites on the SV40 enhancer.

When these promoter sequences were tested in the reverse orientation, we observed that the 524-bp *EgactII* promoter fragment had activity in either direction (pEgII-524CAT and pEgII-524CATR, Figure 2B), as already observed for enhancers and some promoter elements of many cellular, viral and mitochondrial genes (24). The difference in CAT activity between pEgII-524CATR and pCAT Basic was statistically significant, confirming the *EgactII* promoter fragment activity in the reverse orientation.

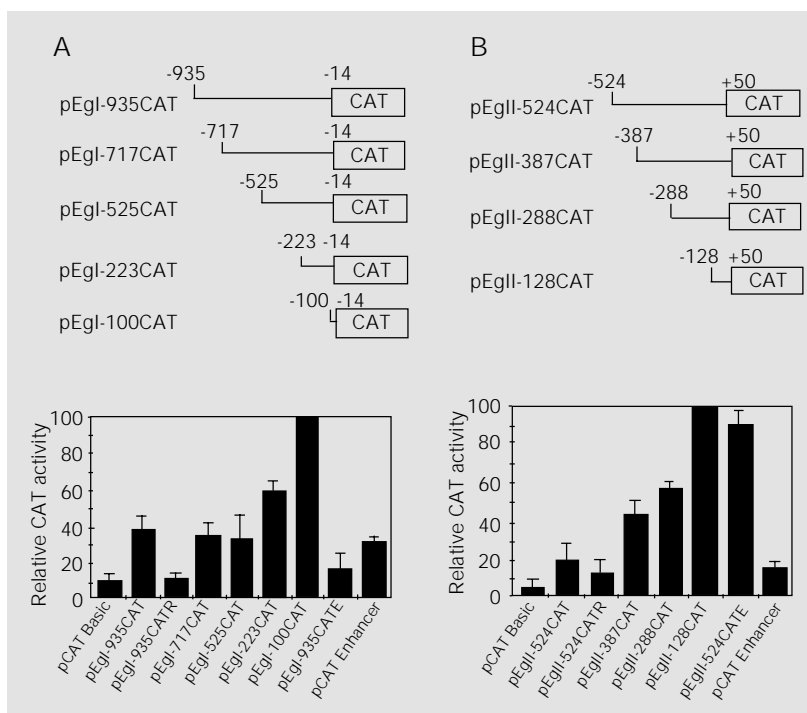


Figure 2 - Transient expression analysis of *EgactI* and *EgactII* promoter-CAT reporter constructs in NIH3T3 cells. *EgactI* (A) and *EgactII* (B) promoter-CAT constructs are schematically represented on the upper part of the figure. The respective transient expression analysis is presented under the promoter-CAT construct schemes. The names of each construct are shown on the left and numbers indicate the relative positions with respect to the ATG codon. Also shown is the transcriptional activity of *EgactI* and *EgactII* promoters in the reverse orientation and their activity in the presence of the SV40 enhancer, designated by the letters "R" (reverse) and "E" (enhancer) in the name of the corresponding constructs. The level of CAT activity was expressed as relative activity compared with the activity of plasmid constructs pEgI-100CAT or pEgII-128CAT (values set at 100). The data represent the mean and standard deviation of 3-5 independent experiments, each performed in duplicate and analyzed by the Student t-test.

Nevertheless, higher activities were observed when this promoter fragment was tested in the original orientation (compare pEgII-524CAT and pEgII-524CATR, Figure 2B). This was also observed for the promoter regions of the *Dictyostelium discoideum* actin gene (24) and the human hypoxanthine-guanine phosphoribosyl transferase gene (25). The bidirectional activity of the *EgactII* promoter may be related to the presence of poly (dT) tracts (26), Sp1 binding sites (27), E-box elements (28) and to the lack of a consensus TATA box sequence (29), already described as characteristic promoter elements with bidirectional activity. In marked contrast, there was no statistically significant difference between pEgI-935CATR and pCAT Basic CAT activities, indicating that the 935-bp *EgactI* promoter fragment presents no bidirectional activity in this system (Figure 2A). The *EgactI* promoter sequence, in contrast to the *EgactII* sequence, has a classical consensus TATA box at position -87, which could have a directional role for transcription, as evidenced by other authors (23).

In order to identify the relevant promoter regions containing regulatory elements involved in promoter activity, we constructed a series of actin promoter 5'-deletion mutants-CAT reporter constructs. The 5'-deletions of the *EgactII* promoter progressively increased promoter activity to a level 5.7 times higher than that obtained with the intact construct (Figure 2B). This increase in promoter activity suggests that the region 524 bp upstream from the ATG contains at least three contiguous regions bearing additive silencer elements: 1) -524 to -388, 2) -387 to -289 and 3) -288 to -129.

5'-Deletions from -935 to -718 and from -717 to -526 of the *EgactI* promoter did not affect its transcriptional activity, since there was no significant difference between the CAT activities from these constructs and the control (Figure 2), although an E-box and putative binding sites for YY1, Sp1 and Ap1

were present in the deleted regions (see Figure 1). Deletions from -525 to -224 and from -223 to -101 respectively increased CAT activity to a level 1.6 and 2.7 times higher than that obtained with the wild-type promoter construct (compare pEgI-223CAT and pEgI-100CAT with pEgI-935CAT in Figure 2A). These results also suggest the presence of additive silencer elements in the region between -525 and -101. An inhibitory system consisting of additive negative regulatory regions was also found in the promoters of the actin genes *act5C* and *A3* of *Drosophila melanogaster* (30) and *Bombyx mori* (31), respectively. These results suggest that sequences from -525 to -101 (*EgactI*) and from -524 to -129 (*EgactII*) might be involved in the negative regulation of both promoters. Indeed, sequence analysis of both promoter regions (Figure 1) showed the presence of many putative binding sites for the conserved eukaryotic YY1 repressor protein in the deleted regions that contained putative silencer elements. Based on this observation, we considered the possibility that the YY1 repressor protein could be involved in the negative regulation of both promoters. Previous studies have shown that the α -actin gene is regulated by the repressor activity of the YY1 protein (32-35). The YY1 protein seems to present a high degree of evolutionary conservation, particularly striking in the C-terminal half of the molecule that includes the DNA-binding and repressor domains (21). This repressor protein is an important component of the transcription initiation complex and is also involved in many aspects of chromatin organization (36). Considering this conservation, we hypothesized that YY1 or a similar DNA-binding activity present in NIH3T3 nuclear extracts could be involved in the negative regulation of both promoters from *E. granulosus* actin genes.

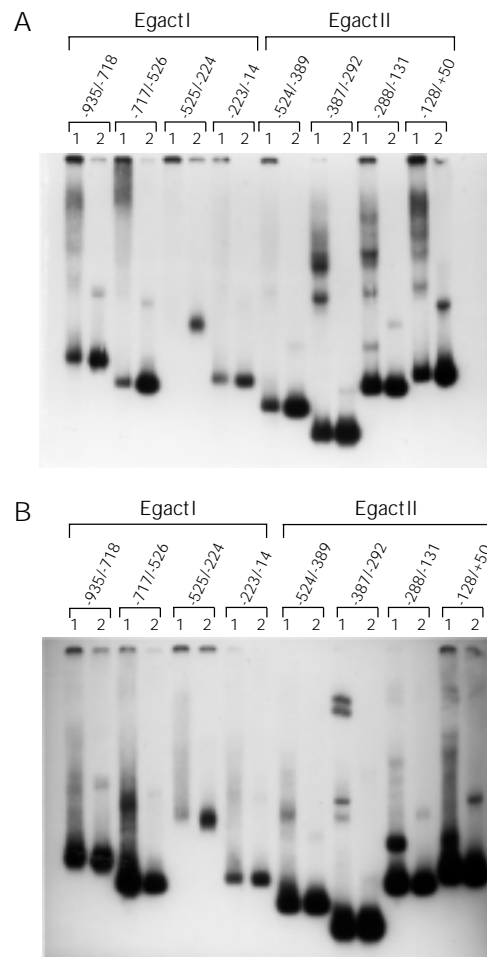
In order to obtain additional evidence about whether the putative YY1-binding sites could be involved in the negative activity of both promoter regions, we performed band

shift assays to verify protein-DNA interactions between NIH3T3 nuclear proteins and these promoter regions (Figure 3A). Protein factors in NIH3T3 nuclear extracts formed DNA-protein complexes with the tested promoter regions (Figure 3A). Assuming that one of the proteins involved in the formation of these complexes was the YY1 transcription factor, we also performed gel shift assays with the human YY1 recombinant protein (Figure 3B). The general patterns of these DNA-protein complexes were similar to those formed with the nuclear extract. Due to this similarity, we hypothesized that the same protein factor might have interacted with the probes. A labeled oligonucleotide containing the YY1 consensus binding site was used as a positive control to test

GST/YY1 recombinant protein and the NIH3T3 nuclear extract. Both extracts formed DNA-protein complexes with the YY1 consensus sequence (data not shown). Taken together, these results seem to suggest that the putative binding sites for YY1 protein found in *EgactI* and *EgactII* promoter sequences may be required for protein-DNA interaction and may be involved in the transcriptional suppression of both actin genes. Further experiments will be necessary to determine whether the factors involved in mediating this repression of *EgactI* and *EgactII* promoters are present in nuclear extracts from *E. granulosus* cells and to test the specificity and identity of binding of the proteins that are forming the retarded complexes with the probes.

The results presented here show, for the first time, the structural and functional characterization of cestode promoter sequences. Our study employing mammalian cells represents the first indication that the basic transcriptional control mechanisms of *Echinococcus* genes operate efficiently in a mammalian context. The successful use of a heterologous transient expression system to examine the transcriptional activity of these two *E. granulosus* actin gene promoters can be mostly attributed to the well-known high degree of conservation of basic transcriptional mechanisms in eukaryotes, especially those related to transcription initiation (37, 38). Our results concerning the functionality of both 5'-flanking regions indicate that conserved transcription factors present in NIH3T3 nuclear extracts are able to recognize, bind and finally activate gene transcription from both promoter regions. Although very little information is available about gene regulation in *E. granulosus*, DNA sequences that code for homeobox and GATA transcription factors have already been isolated from cDNA libraries from this worm (39,40). These sequences show a high level of identity to their mammalian counterparts, indicating that cestode and mamma-

Figure 3 - Binding properties of NIH3T3 protein nuclear extracts and YY1 recombinant protein in *EgactI* and *EgactII* promoter regions. *EgactI* and *EgactII* promoter regions were retarded by NIH3T3 protein nuclear extracts and by YY1 recombinant protein. *EgactI* and *EgactII* [³²P]-labeled promoter fragments (lanes 2) were used as probes in a gel mobility shift assay experiment in the presence of 5 µg of NIH3T3 nuclear extract (panel A, lanes 1) or GST/YY1 recombinant protein (panel B, lanes 1) and resolved on a native 6% polyacrylamide gel. Four delimited promoter regions (*EgactI* on the left and *EgactII* on the right) are shown on the top of the figure. The intense band at the bottom of each lane corresponds to the probe run in the absence of protein.



lian cells present similar transcription factors involved in the basic mechanisms of transcription regulation. For these reasons, the use of a heterologous system to test cestode promoter activity can be of wide interest to elucidate many basic and conserved aspects of transcriptional regulation in these organisms. These studies can also contribute to the efforts to better understand the basic molecular mechanisms involved in the control of gene expression in *E. granulosus*.

Acknowledgments

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