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Analysis of the 170-kDa lectin gene promoter of *Entamoeba histolytica*

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Abstract

The promoter region driving the gene for the 170-kDa heavy subunit of the *Entamoeba histolytica* galactose-inhibitable lectin was analysed by transient transfection using the chloramphenicol acetyltransferase gene as reporter. S1 mapping confirmed our previous notion that the promoter is located within a 1.35-kb intergenic sequence preceding the structural lectin gene. Transcripts derived from the chloramphenicol acetyltransferase gene of transfected trophozoites were found to be polyadenylated and the transcriptional start mapped to a position similar to that of the wild-type lectin gene. By deletion analysis the entire promoter was restricted to a fragment covering about 550 bp upstream from the start of transcription. On the other hand, residual promoter activity required a sequence of about 140 bp only, encompassing a newly identified CCAAT-box like element around position −100, as well as the amebic specific TATA-box. This 140-bp fragment as well as a stretch of 15 bp, which is located some 100 nt further upstream, were found to be conserved within the 5′ noncoding region of a second *E. histolytica* lectin gene. Point-mutation analyses indicated that the 15-bp fragment, the likely CCAAT-box, as well as the TATA-box are required for full promoter activity.

Keywords: *Entamoeba histolytica*; Amebiasis; Promoter analysis; Transfection; CAT assay

1. Introduction

Despite the medical importance of the enteric protozoan parasite *Entamoeba histolytica* little is known about gene organization and control of gene expression in this pathogen. On the basis of a few genomic sequences so far analysed, recent studies indicated that transcriptional units are closely linked and located unidirectional within the ameba genome [1,2]. Preliminary investigations of two intergenic regions by nuclear run-on or transient transfection assays suggested that transcription in *E. histolytica* is monocistronic rather than polycistronic [2,3]. In addition, the comparison of various *E. histolytica* gene sequences revealed some peculiarities for the gene organisation of the parasite: (*i*) with two exceptions so far known [4,5], coding sequences are not interrupted by introns, (*ii*) 5′ and 3′ untranslated

Abbreviations: CAT, chloramphenicol acetyltransferase; PE, primer extension; S1, S1 nuclease.

Note: The nucleotide sequence data reported in this paper have been submitted to the EMBL Data Library with the accession numbers X70850 and X83757.

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regions are rather short, comprising only few nucleotides, (*iii*) transcription starts at the consensus sequence ATTCA or ATCA, and (*iv*) an unusual TATA-motif comprising the sequence TATTTAAA is located about 30 nt upstream from the start of transcription in most *E. histolytica* genes [2]. No further elements which might be important to drive gene expression in *E. histolytica* have been identified by sequence homology searches.

The recent development of transient transfection systems in *E. histolytica* provide the basis for the in vivo analysis of promoter sequences [3,6]. The first promoter positively identified in *E. histolytica* was found to be located within a 1.35-kb intergenic region driving the gene encoding the 170-kDa heavy subunit of the galactose-inhibitable lectin [2].

Here we report on a detailed genetic dissection of the lectin gene promoter region, indicating that novel *cis*-acting sequences are involved in the generation of lectin transcripts in *E. histolytica*.

2. Materials and methods

2.1. Cultivation of parasites

For all experiments, the axenically cultured *E. histolytica* isolate HM-1:IMSS was used, grown in TYI-S-33 medium [7].

2.2. Construction of promoter mutants

Constructs were made and sequenced by standard methods. All clones are derivates of expression plasmid pL5′A3′CAT [3]. The plasmid contains a fragment of 1430 bp upstream from the lectin translation initiation ATG, followed by the CAT coding sequence and 600 bp of an *E. histolytica* actin gene sequence downstream from the TAA stop codon. 5′ or internal deletion were made using appropriate restriction sites or by PCR amplification of respective fragments and cloned in front of the CAT coding region (see Fig. 4). Point mutations were generated by site-directed mutagenesis of plasmid p550L5′A3′CAT using the Sculptor in vitro mutagenesis system (Amersham). Oligonucleotide primers being used are: 5′-GTT TCA TAA TGA CCA TAT TTT CGA TGA TG for mut1, 5′-CAA GAG AGT TTA TTT GAC CAT TAT TAA CAC C for mut2, 5′-CAT TGA GTT GTG ACC ATT ATC TTA TTT TTC C for mut3 and 5′-CAT GGT ACC GAC CTT TTT AAA GTT CAT TGA G for mut4 (see Fig. 5).

2.3. E. histolytica transfection

Transfections were performed as previously described [3] with the minor modification that instead of one, two subsequent pulses were introduced, each with an exponential discharge of 3000 V cm^{-1} at a capacitance of 25 μF using the Bio-Rad Gene Pulser. We generally transfected 2×10^6 trophozoites either with 50 μg circular plasmid DNA per cuvette for subsequent CAT enzyme assays, which were performed 48 h after transfections or with 200 μg DNA per cuvette for subsequent RNA analysis isolated 24 h after transfection. CAT activity was analysed by the two-phase diffusion assay [8] using 10 μg of trophozoite extracts and [^{14}C]butyryl CoA (Dupont-NEN). CAT activity was determined always in the linear range of the assay. Each construct was assayed at least five times and all assays were performed in duplicate.

2.4. RNA analysis

RNA was isolated from trophozoites by ultracentrifugation through a CsCl cushion [9] and analysed by standard procedures [10]. For Northern blot analysis, 10 μg of total, 10 μg of poly(A)$^-$ and 2 μg of poly(A)$^+$RNA, respectively, were separated on a 1% agarose-formaldehyde gel, transferred to nylon membranes and hybridised with random primed probes. For primer extension studies, 1 pmol of end-labelled oligonucleotide CAT-AS19 (5′-CCG TAA TAT CCA GCT GAA C) complementary to nt 109–127 of the CAT coding sequence and 3 μg of poly(A)$^+$ trophozoite RNA were used. Primer extension products were tailed with dGTP and amplified by PCR using CAT-AS19 and an oligo C_{16}-primer [11]. Amplified DNA fragments were cloned into pBS and sequenced. For S1 mapping, two double stranded probes, a 526-bp *Mae*III-*Sca*I fragment and a 667-bp *Eco*RV-*Nco*I fragment (Fig. 1), were generated by endonuclease digestion of genomic clone gEh-170/1 [2]. The 526-bp fragment was radiolabelled by a

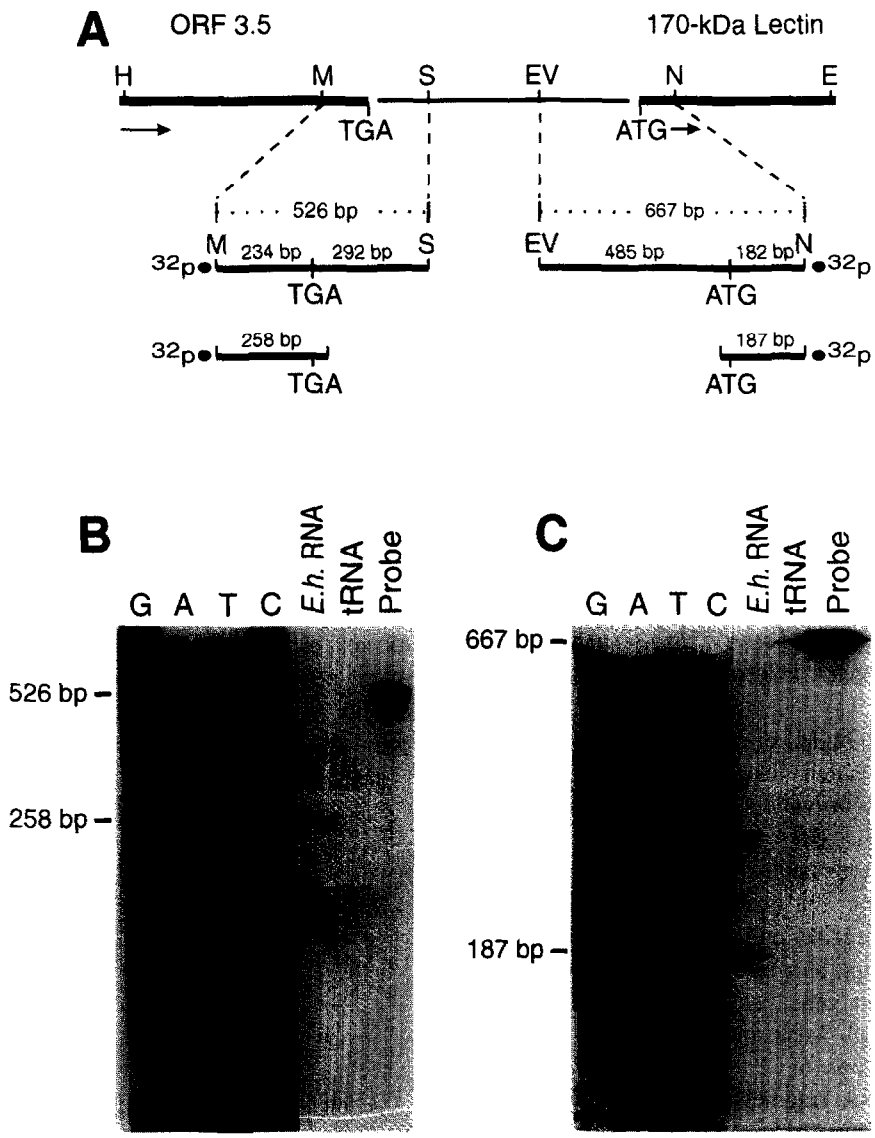

Fig. 1. S1 analysis to map the polyadenylation site of the ORF 3.5 gene, as well as the transcription initiation site of the lectin gene within the 1.35-kb intergenic region of gEh-170/1. (A) Shown is a schematic depiction of the genomic organization and the orientation of the ORF 3.5 and the 170-kDa lectin genes. Relevant restriction sites are indicated: E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; M, *Mae*III; N, *Nco*I; S, *Sca*I. (B,C) Two double-stranded probes, a 526-bp *Mae*III-*Sca*I fragment and a 667-bp *Eco*RV-*Nco*I fragment were generated by endonuclease digestion of genomic clone gEh-170/1. The 526-bp fragment was radiolabelled by a fill-in reaction at the *Mae*III site, whereas the 667-bp fragment was radiolabelled by polynucleotide kinase at the *Nco*I site. Each of the labelled fragments was hybridised to 100 μg of total cellular trophozoite RNA (E.h.RNA) or 100 μg of yeast tRNA (t-RNA). Upon digestion with S1 (37°C and 250 U ml^{-1} of enzyme for 60 min) products were analysed by gel electrophoresis. The sizes of the products were determined by comparisons with respective sequencing reactions.

fill-in reaction at the *Mae*III-site and hybridised to RNA at 37°C, whereas the 667-bp fragment was radiolabelled by polynucleotide kinase at the *Nco*I-site and hybridised to RNA at 42°C. Hybridization was performed overnight using 2×10^5 cpm of each probe and 100 μg of total, cellular trophozoite RNA

or 100 μg of yeast tRNA. S1 digestion was carried out at 37°C for 60 min with 250 U ml^{-1} of S1.

2.5. Cloning of lectin genes

A genomic λZAP library derived from *E. histolytica* isolate HM-1:IMSS [2] was screened under low stringency with a 5′ portion of ZAP-170/4, an almost full-length cDNA coding for one of the *E. histolytica* 170-kDa lectins [12]. Seven of the hybridizing phages were purified, and the plasmids were released according to the instructions of the manufacturer (Stratagene). Inserts were analysed by restriction mapping and DNA sequencing.

3. Results

3.1. Mapping of end and start of transcription within the 1.35-kb intergenic fragment

By Northern blot and nuclear run-on analyses, we recently demonstrated that the entire lectin gene promoter of *E. histolytica* should be placed within a 1.35-kb intergenic DNA fragment, located downstream from a long open reading frame (ORF 3.5) and upstream from the lectin coding region [2]. To determine the precise polyadenylation site of the ORF 3.5 transcript, as well as the start of transcription of the lectin gene, S1 protection assays were performed (Fig. 1). Taken into account that 3′ and 5′ untranslated regions of *E. histolytica* mRNAs are rather short, DNA fragments presumably spanning either the end of transcription of the ORF 3.5 gene or the start of transcription of the lectin gene were isolated. Each of the two fragments were hybridised to *E. histolytica* RNA and subsequently subjected to S1-nuclease digestions. Protected fragments were obtained exceeding the stop codon of the ORF 3.5 gene by 24 nt (Fig. 1B) and the translation initiation ATG of the lectin gene by 5 nt (Fig. 1C), respectively. The results are in agreement with previous findings in that transcription terminates and reinitiates within the 1.35-kb intergenic fragment, and that the transcriptional start site of the lectin gene maps within the ATTCA-consensus motif, few nucleotides upstream from the translation initiation ATG [2,12].

3.2. RNA analysis of transfected E. histolytica

Trophozoites were transfected with hybrid construct pL5′A3′CAT previously used for successful expression of chloramphenicol acetyltransferase (CAT) in *E. histolytica*. This construct comprises the CAT coding sequence flanked by 1430 bp of the lectin gene upstream region and 600 bp of an actin gene downstream region [3]. Northern blot analysis using total as well as poly(A)$^-$ or poly(A)$^+$RNA of transfected cells revealed a single, polyadenylated CAT-specific transcript with an estimated size of about 700 bp (Fig. 2). The 5′ end of CAT RNA in pL5′A3′CAT-transfected trophozoites was determined by primer extension analysis, using an antisense oligonucleotide complementary to nt 109–127 of the CAT coding region. A single extension product was obtained, exceeding the CAT translation initiation ATG by 8 nt (Fig. 3A). Amplification of the extension product by anchored PCR and subsequent sequencing proved the start of transcription within the ATTCA-motif of the flanking lectin gene sequence (Fig. 3B), indicating similar positions for

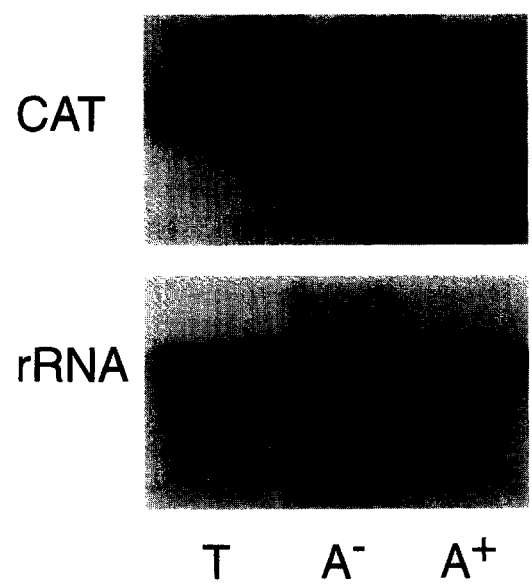

Fig. 2. Northern blot analysis of RNA from trophozoites transfected with pL5′A3′CAT. A formaldehyde gel was loaded either with 10 μg of total RNA, 10 μg of poly(A)$^-$ RNA or 2 μg of poly(A)$^+$ RNA, blotted and sequentially hybridised with a purified CAT coding sequence and an *E. histolytica* rRNA gene probe.

the start of transcription for the CAT hybrid construct and the wild-type lectin gene.

3.3. Deletion analysis of the lectin gene promoter

In order to define the promoter region of the *E. histolytica* lectin gene more narrowly, the 1430-bp fragment of pL5′A3′CAT was modified either by inserting it in opposite orientation or by creating various 5′ or internal deletions. As shown in Fig. 4, comparison of CAT activity in trophozoites transfected with pL5′A3′CAT and the various deletion constructs revealed: (*i*) Complete loss of CAT expression when the 1430-bp fragment was used in opposite orientation or deleted down to 70 bp; (*ii*) full CAT activity with 5′ deleted fragments covering at least 550 bp; (*iii*) reduction of CAT activity by 50% with fragments covering 485 bp or 200 bp and (*iv*) further reduction of CAT activity down to 13% using a 140-bp fragment. The results suggest that the 550-bp fragment most likely contains the complete lectin gene promoter. In addition, two regions seem

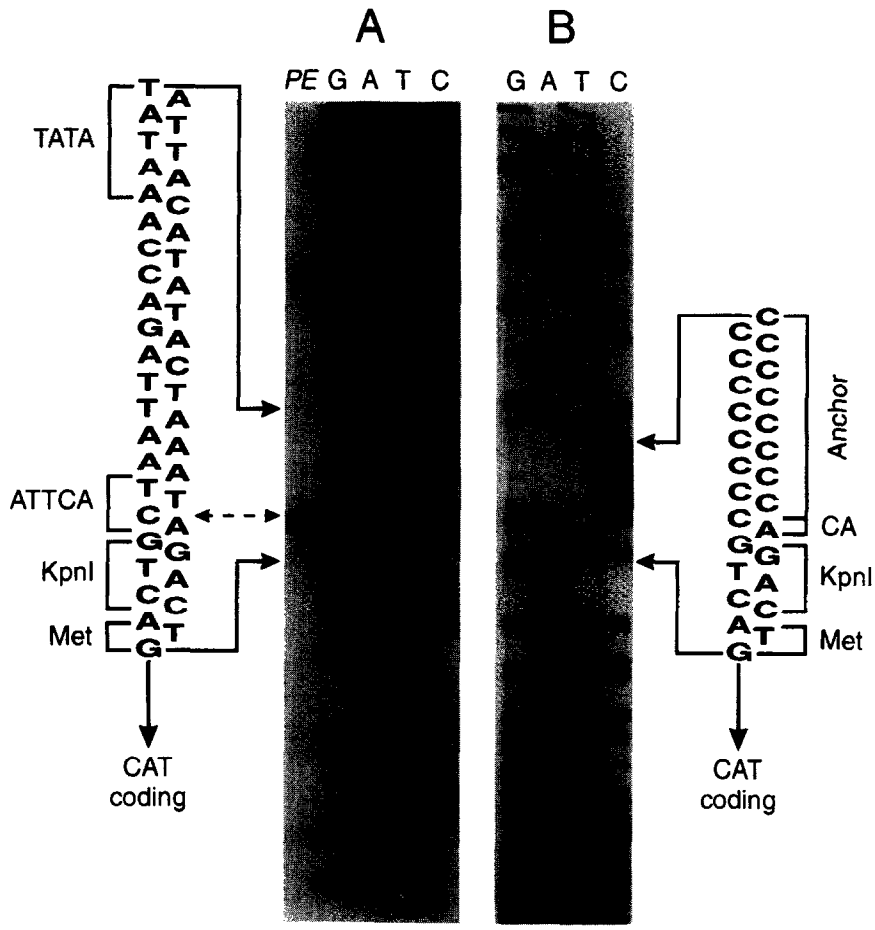

Fig. 3. Primer extension to map the start of CAT-RNA transcripion in pA5′A3′CAT transfected trophozoites. (A) An end-labelled primer complementary to nt 109–127 of the CAT coding sequence was hybridized to 3 μg of poly(A)$^+$ trophozoite RNA. Extension products (PE) were analysed by gel electrophoresis and compared with a sequencing reaction of pL5′A3′CAT using the same primer. (B) The primer extended product was tailed with dGTP, amplified by anchored PCR technology [11], cloned and sequenced. The likely TATA-box, the ATTCA-motif at start of transcription, the oligo C-anchor sequence, the *Kpn*I site used to connect the lectin upstream sequence with the CAT-coding sequence, as well as the CAT gene translation initiation site (MET) are indicated.

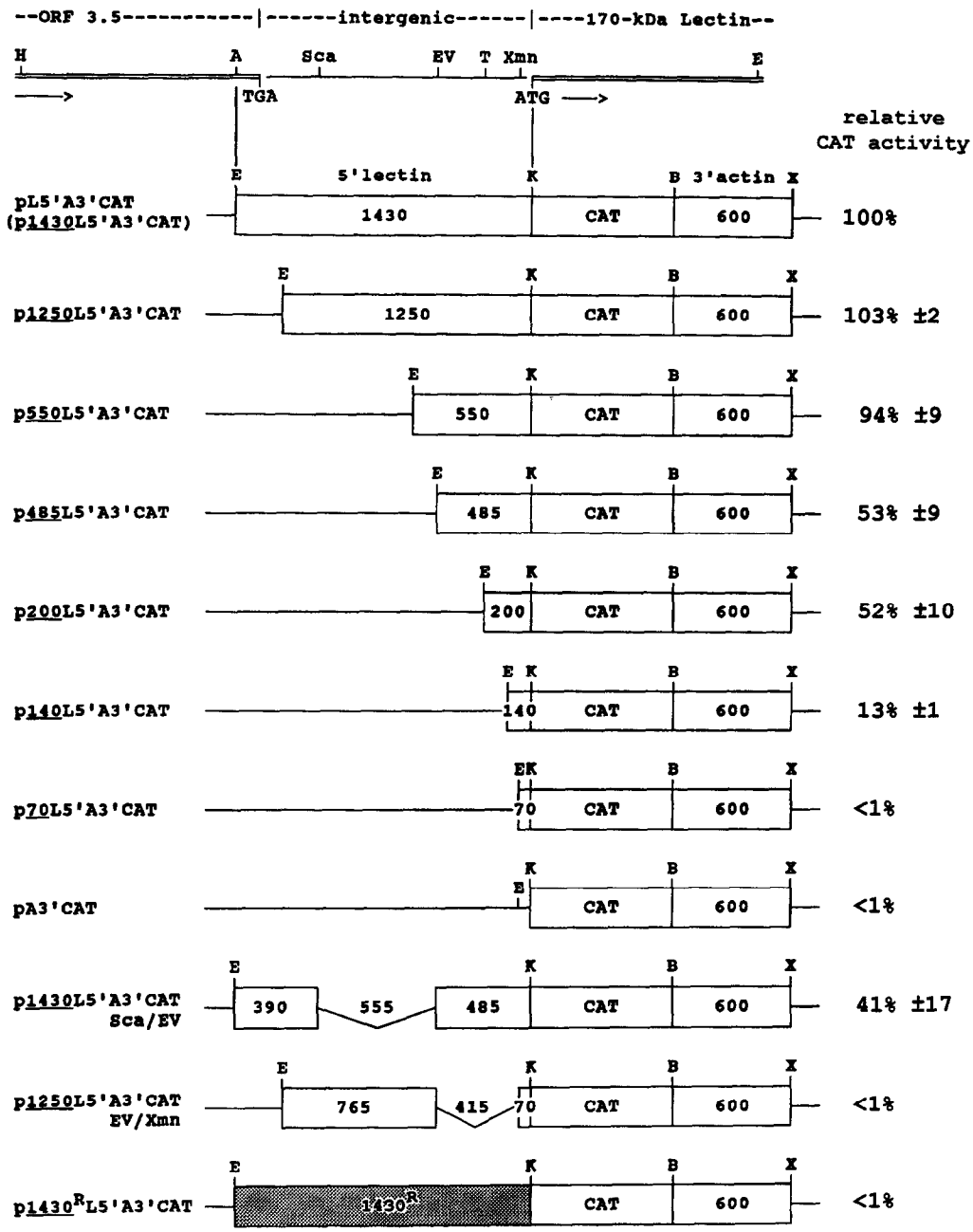

Fig. 4. CAT gene expression in *E. histolytica* trophozoites transfected with 50 μg of various hybrid constructs for the analysis of the lectin gene promoter. Shown are schematic depictions of the 1.35-kb intergenic region comprising the lectin gene promoter, as well as those of the various mutant constructs used for transfection. On the right is shown the CAT activity produced in transfected cells, calculated relative to the CAT activity obtained with control clone pL5′A3′CAT. All mutants were derived from pL5′A3′CAT [3]. This plasmid comprises 1430 bp of the 5′ lectin sequence preceding the translation initiation ATG (5′lectin), the CAT coding region from ATG to the TAA stop codon (CAT) followed by the noncoding downstream sequence of an *E. histolytica* actin gene (3′actin). In p1430^{R}L5′A3′CAT, shown at the bottom, the 1430 bp intergenic fragment was introduced in opposite orientation. Relevant restriction sites used to create several of the 5′ and internal deletions and for subcloning the different fragments are indicated. A, *Alu*I; B, *Bam*HI; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; Sca, *Sca*I; T, *Taq*I; X, *Xba*I; Xmn, *Xmn*I. The length of the different fragments is given in bp.

to be important for full promoter function located between positions −70 and −200 and between −485 and −550, respectively.

3.4. Sequence comparison of two different E. histolytica lectin genes

Previous investigations demonstrated that a family of at least three different genes is coding for the 170-kDa lectin or closely related proteins in at least the *E. histolytica* isolate HM-1:IMSS [12,13]. By screening a genomic library derived from the *E. histolytica* isolate HM-1:IMSS using a fragment covering the region coding for the N-terminal portion of the lectin, several hybridising phages were detected of which seven were purified and further analysed. Independent overlapping clones were identified by analysis with several restriction endonucleases. Overlapping regions derived from at least two independent clones were sequenced. The clones were classified as members of two different genomic loci. Two of them were found to represent gEh-170/1, the lectin gene isolated previously [2], whereas all of the remaining 5 were derived from a different genomic locus, designated gEh-170/2. About 5 kb of gEh-170/2 were sequenced comprising the majority of the coding sequence and of about 1700 bp of the 5′ upstream region. In comparison to gEh-170/1, the nucleotide sequence of gEh-170/2 differed by about 10% within the coding region and by about 40% within the 140 bp preceding the translation initation site. Further upstream, no overt homology was found between the two sequences, except a fragment of 15 bp located around position −520 in gEh-170/1 and around position −390 in gEh-170/2, respectively, with sequence identity of 87% (13 out of 15 nt) (Fig. 5).

Besides the TATA-box motif a second conserved consensus sequence was identified by comparing the two upstream sequences with those of various *E. histolytica* genes (Fig. 6). This motif comprises the sequence TCCAAAA located about 100 nt upstream from the start of transcription, reminiscent of the CCAAT-box of gene promoters in higher eukaryotes.

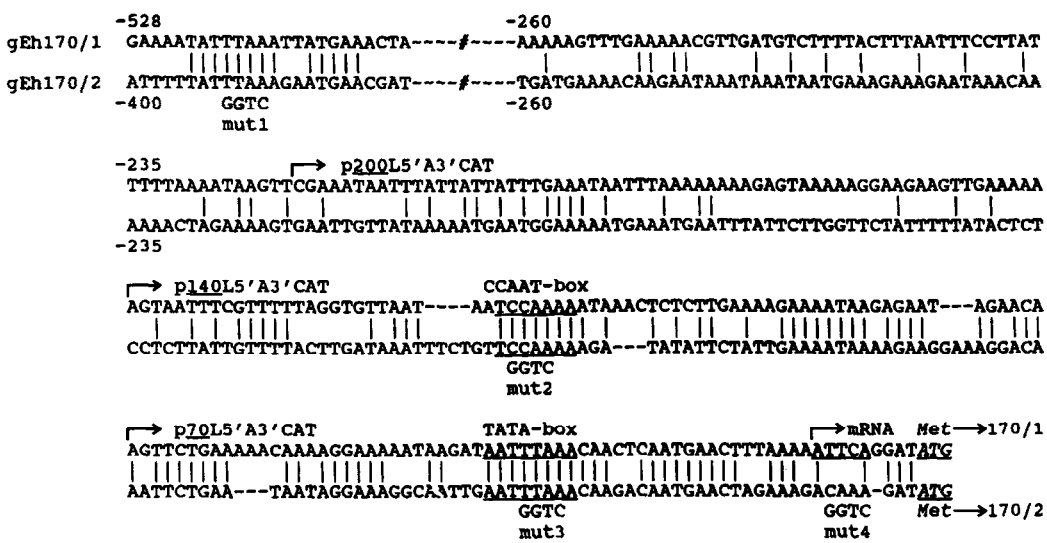

Fig. 5. Comparison between the 5′ upstream sequences of gEh170/1 and gEh170/2. Shown are the 260 nt preceding the translation initiation ATG of both genes, as well as the homologous sequences around position −520 and −390, respectively. Identical nucleotides are indicated by vertical dashes. For optimal alignments, gaps (–) were introduced into the sequences. The 5′ ends of deletion constructs p200L5′A3′CAT, p140L5′A3′CAT and p70L5′A3′CAT, as well as the transcriptional start site are indicated by arrows. The likely CCAAT- and TATA-boxes as well as the ATTCA-motif at start of transcription are underlined. The positions of the GGTC-motif used to alter several promoter elements are indicated below the sequences. Note: Within the expression plasmids the stretch of 4 nt (GGAT) between the ATTCA-motif and the translational initiation ATG is replaced by the sequence GGTACC to create a *Kpn*I restriction site which was used to link the different promoter mutants to the CAT coding sequence (see Fig. 3).

	-101		
gEh170/1	TCCAAAA	[this paper]	
	-98		
gEh170/2	TCCAAAA	[this paper]	
	-100		
gEhAP	TCTAAAA	[2]	
	-96		
gEhFeSOD	TCCGAAA	[2]	
	-111		
gEhActin1	TCCAAAA	[14]	
	-84		
gEhSREHP	TTCAACT	[15]	
	-105		
gEhFerredox	TTCAAAA	[16]	
	-95		
gEh34kDa	TTCAATA	[17]	
consensus	TCCAAAA		

Fig. 6. CCAAT-box like sequence motifs within the 5′ flanking regions of various *E. histolytica* genes. The 7-bp sequences and their positions relative to the start of transcription are indicated. Numbers in brackets refer to the corresponding references.

3.5. Functional analysis of sequences conserved within the 5′ flanking regions of the two lectin genes

To investigate the functional importance of sequence motifs found to be conserved within the 5′ flanking sequences of the two 170-kDa lectin genes, point mutations were generated within the 550-bp promoter fragment. Each of the mutants was created by site-directed mutagenesis replacing 4 consecutive nucleotides by the sequence GGTC, to alter either the 15-bp motif at position −520 (mut1), the likely CCAAT-box (mut2), the likely TATA-box (mut3) or the ATTCA-motif at the start of transcription (mut4) (Fig. 5). Trophozoites were transfected with a CAT hybrid construct containing the 550-bp wild-type promoter sequence or with constructs containing the various point mutations within the 550-bp fragment. Determination of CAT activity revealed that in comparison with the wild-type sequence, transfection of each of the mutant constructs resulted in a significant reduction of CAT expression (Table 1), suggesting that all the conserved sequence motifs are required for efficient expression of the 170-kDa lectin gene.

Table 1
CAT activity of *E. histolytica* trophozoites transfected either with p550L5′A3′CAT or with the various mutants thereof (mut1–4)

Construct	Relative CAT activity (%)
p550L5′A3′CAT	100
mut1 (15 bp)	43 ± 7 [a]
mut2 (CCAAT)	52 ± 13
mut3 (TATA)	20 ± 7
mut4 (ATTCA)	32 ± 12

[a] CAT activity is expressed relative to the activity of cells transfected with p550L5′A3′CAT.

4. Discussion

As a first approach to gain insight into the structural requirements driving gene expression in *E. histolytica*, we took advantage of the recent development of amebal transient transfections to analyse the 170-kDa lectin gene promoter of the parasite. S1-analysis confirmed our previous observation that this promoter is located within the 1.35-kb intergenic region preceding the structural lectin gene [2].

The CAT RNA produced after transfection was found to be polyadenylated and comprises a 5′ untranslated sequence of 8 nt only. In addition, start of CAT-RNA transcription mapped to the ATTCA consensus motif within the lectin 5′ sequence, at a position similar to the start of transcription of the wild-type lectin gene. The fact that only a single primer extension product was obtained by analysing CAT-RNA of transfected trophozoites indicates that there is no additional relevant transcription initiaton site further upstream within the lectin 5′ sequence or within the vector DNA. The latter is also supported by the finding that transfection with a construct in which the lectin 5′ sequence was introduced in opposite orientation failed to generate CAT expression. Taken together, these results strongly suggest that the lectin gene upstream sequence used within the CAT hybrid construct is able to drive RNA expression in a similar way as it does within the wild-type lectin gene. Therefore, transient transfection assays using this hybrid construct seem to be a suitable tool to study the 'anatomy' of the lectin gene promoter in *E. histolytica*. However, we have not excluded the possibility that sequences of the lectin coding region or of the lectin 3′ flanking sequence do influence the promoter activity, since those sequences were not included into the hybrid constructs.

In addition, we did not monitor transfection efficiency of the various promoter mutants, which could be done for instances by co-transfection of a second, independent reporter gene. Therefore, there may be certain limits in comparing the results obtained with the different constructs. However, the high reproducibility of CAT activity in muliple series of duplicate assays using two or three different DNA preparations of each promoter construct suggests validity of the results presented.

Our deletion analysis of the lectin 5′ sequence revealed that about 550 bp preceding the transcription initiation site are required for full promoter activity. The smallest fragment covering residual promoter activity was found to comprise 140 bp only, encompassing the likely CCAAT- as well as the likely TATA-box. Interestingly, these 140 bp were found to be well conserved within the 5′ flanking region of a second lectin gene, suggesting that this region resembles the core promoter of the lectin gene. Mutation analysis confirmed the importance of the likely CCAAT- and TATA-boxes for promoter activity, as well as of a sequence further upstream that might function as an enhancer element. So far, only the TATA-box like sequence of *E. histolytica* has been shown to bind nuclear proteins [2]. Whether the other identified elements represent binding sites for putative transcription factors remains to be determined. In addition, a more precise mutation analysis of the lectin gene promoter has to be performed to rule out the possibility that additional *cis*-acting elements so far undetected by homology search are also involved in driving lectin gene expression. Such elements are at least expected within the stretch of 60 nt between position −140 and −200, since deletion of this sequence resulted in a significant decrease in CAT activity.

However, even when the analyses presented here are the first of its kind performed in *E. histolytica*, the results strongly suggest that gene promoters of this protozoon are composed like those of other eukaryotes comprising various cis-acting elements required for full promoter function.

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