The Novel Core Promoter Element GAAC in the \textit{hgl5} Gene of \textit{Entamoeba histolytica} Is Able to Direct a Transcription Start Site Independent of TATA or Initiator Regions* \\

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\textit{Entamoeba histolytica}, an enteric protozoa, is the third leading parasitic cause of death worldwide. Investigation of the transcriptional machinery of this eukaryotic pathogen has revealed an unusual core promoter structure that consists of nonconsensus TATA and initiator regions and a novel third conserved core promoter sequence, the GAAC element. Mutation of this region in the \textit{hgl5} promoter decreases reporter gene expression and alters the transcription start site. Using positional analysis of this element, we have now demonstrated that it is able to direct a new transcription start site, 2–7 bases downstream of itself, independent of TATA and Inr regions. The GAAC region was also shown to control the rate of transcription via nuclear run on analysis and an amebic nuclear protein was demonstrated to specifically interact with this sequence. This is the first description in the eukaryotic literature of a third conserved core promoter element, distinct from TATA or initiator regions, that is able to direct a transcription start site. We have formulated two models for the role of the GAAC region: (i) the GAAC-binding protein is a part of the TFIID complex and (ii) the GAAC-binding protein functions to “tether” TATA-binding protein to the TATA box.

\textit{Entamoeba histolytica} is a single cell eukaryote that causes invasive amebic colitis and liver abscess. Infection with this organism is an important contribution to morbidity and mortality in developing countries, and worldwide it is the third leading parasitic cause of death. During its life cycle \textit{E. histolytica} undergoes developmental changes such as transformation from the cyst to trophozoite and adaptation from an anaerobic to aerobic environment upon invasion. How \textit{E. histolytica} regulates these events is not understood, although regulation of transcription is likely to be an important mechanism of this control. Recently two papers have described transcriptional control of a drug resistance gene in \textit{E. histolytica} (1, 2), thus demonstrating a relationship between pathogenesis and regulation of transcription.

At a molecular level, little is known about the control of gene expression in this organism. As an early diverging member of the eukaryotic tree, \textit{E. histolytica} has many unusual characteristics with regard to gene organization. These include a genome that is AT-rich (67% within coding regions and 78% overall) (3, 4) and compact (1.5 \times 10^7 \text{bp}) (5) and an RNA polymerase II that is resistant to \alpha-amanitin (6). A putative \textit{E. histolytica} TBP has been reported (GenBank\textsuperscript{TM} accession number Z48307) that has significant sequence divergence from the TBP of \textit{Drosophila melanogaster}, \textit{Caenorhabditis elegans}, and \textit{Plasmodium falciparum} (7). In addition, we have recently found that yeast TBP does not bind to the TATA box of the \textit{E. histolytica} \textit{hgl5} gene.\textsuperscript{2} It has been shown that amebic promoter sequences do not function in a mammalian system and that viral promoters (cytomegalovirus, human immunodeficiency virus long terminal repeat, the simian virus 40) and promoters from other systems (\textit{Dictyostelium}) are nonfunctional in amebic trophozoites (8, 9). Thus, it would appear that species-specific transcription factors may be utilized in amebic gene expression.

The core promoter region in metazoans is the target of a variety of regulatory proteins that work in concert to direct the complex mechanisms of transcriptional control. Transcription of mRNA relies on the assembly of RNA polymerase II and a variety of other transcription factors (TFIID, TFIIA, TFIIB, TFIIF, and TFIH) into a stable and functional preinitiation complex (10). The preinitiation complex may assemble in a sequential manner on the DNA sequence of the core promoter, or a “holoenzyme” complex may form, which then binds specifically to the core promoter region (11). Both the TATA box and Inr regions appear to direct the formation of the preinitiation complex, control the site of transcription initiation, and regulate activation by upstream activator proteins (12). Recently, variations to this classic core promoter architecture have been described. A third conserved core promoter element has been identified in a subset of TATA-less promoters of \textit{Drosophila} (13), in the human angiotensinogen gene (14), and the core promoter of the hepatocyte growth factor (15). A consensus element recognized by TFIIB and immediately adjacent to and upstream of TATA has also been described (16). Thus, there appears to be an emerging body of literature on an alternative core promoter architectural structure.

We have shown recently that the \textit{E. histolytica} core promoter contains three elements that control the site of transcription initiation (9). The amebic TATA (GTATTTTAA/G/C) and Inr (AAAAATTCA) elements appear to function in a classic manner despite their sequence divergence from metazoans (9). A unique third core promoter element GAAC (GAAC\textsuperscript{5}) was identified in 31/37 protein-encoding \textit{E. histolytica} genes (17). It has a variable location between the TATA and Inr sequences, a

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\textsuperscript{1} The abbreviations used are: bp, base pair; TBP, TATA-binding protein; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay(s); Inr, initiator; luc, luciferase; GBP, GAAC-binding protein; TF, transcription factor.

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\textsuperscript{2} U. Singh, J. Rogers, and W. A. Petri, Jr., unpublished results.
characteristic not defined previously for core promoter transcription elements in metazoa. Mutation of this region in the hgl5 gene promoter had a greater effect on gene expression and selection of the site of transcription initiation than mutation of either the TATA or Inr regions (9). These characteristics of the GAAC region would seem to usurp the dominant role of the TATA element in transcriptional control in E. histolytica.

Our goal was to determine how the GAAC element in the hgl5 gene of E. histolytica regulates the rate and site of transcription initiation. This was undertaken by performing positional analysis of the GAAC region. We determined that it affected the rate of transcription, did not function as an enhancer element, and was able to direct a site of transcription initiation independent of a TATA or Inr element. An amebic nuclear protein was demonstrated to specifically interact with this DNA sequence as shown by EMSA. Based on the data, we have formulated a model for the role of the GAAC region in transcriptional control in E. histolytica.

MATERIALS AND METHODS

Cultivation of E. histolytica and Stable Transfection—E. histolytica strains of T. cruzi were transfected in TYI-S-33 medium containing penicillin (100 units/ml) (Life Technologies, Inc.) and streptomycin (100 μg/ml) (Life Technologies, Inc.) (18). Stable transfection was performed using two promoter vectors (5' hgl5-luciferase-hgl3' and 5' act-neo'-act 3') described previously (19, 20). The trophozoites were maintained at 24 °C and 50% relative humidity, which were extracted by digestion of the plasmids pTP.4i and pTP.GAAC-CLA (9). Construction of the plasmid pTP.4i was undertaken on the plasmids pTP.4i and pTP.GAAC-CLA (9). Construction of the plasmid pTP.4i was undertaken on the plasmids pTP.GAAC-CLA the 5' region fused to the neomycin drug selection gene (9, 17, 20). In the hgl5 gene of E. histolytica has the GAAC region mutated to a GAACT core promoter region of the hgl5 gene (9). The primer used to generate this was 5'-GAACAAGAGTTAGAAATAGGAAAGGT-3' (italicics). This oligonucleotide was used for the gel shift assays not to contain a TATA region. An E. histolytica hgl5 promoter sequence (5'-AATTGCTATACGGGTACCAGCGCTACAAGTGGAATGAG-3') with a mutated GAAC region (bold) was used as double-stranded competitors for the gel shift assay. The probe was purified by a polyacrylamide gel electrophoresis procedure (24). Modifications on this method included the incubation of the polyacrylamide gel section containing the probe at 37 °C overnight in elution buffer (TE (10 mM Tris, 1 mM EDTA, pH 8.0) with 100 mM NaCl) prior to centrifugation for 30 min at 10,000 g. The supernatant was then saved and the pellet washed with an additional 400 μl of elution buffer and re-centrifuged. The two supernatants were combined, the pellet discarded, and the filtered probe was ethanol-precipitated at -70 °C. One pmol of this purified probe was labeled with [α-32P]dATP using the Klenow fragment of DNA polymerase I and purified using a monoclonal antibody to a NuClump column (Stratagene, La Jolla, CA).

RESULTS

Positional Analysis of the GAAC Element as Assessed by Reporter Gene Expression—Previously we had shown that mutation of the GAAC region in the core promoter of the hgl5 gene of E. histolytica resulted in decreased reporter gene expression and mRNA levels (9). Since the GAAC element has been shown to affect the rate of transcription, did not function as an enhancer element, and was able to direct a site of transcription initiation independent of a TATA or Inr element. An amebic nuclear protein was demonstrated to specifically interact with this DNA sequence as shown by EMSA. Based on the data, we have formulated a model for the role of the GAAC region in transcriptional control in E. histolytica.

Plasmid Construction—Positional analysis of the GAAC region was undertaken on the plasmids pTP.4i and pTP.GAAC-CLA (9). Construction of the pTP.4i plasmid was described earlier and consists of a 272-bp hgl5 upstream region fused to a luciferase reporter gene and the 5' act region fused to the neomycin drug selection gene (9, 17, 20). The trophozoites were maintained at 24 °C and 50% relative humidity, which were extracted by digestion of the plasmids pTP.4i and pTP.GAAC-CLA the 5' region fused to the neomycin drug selection gene (9, 17, 20). In the hgl5 gene of E. histolytica has the GAAC region mutated to a GAACT core promoter region of the hgl5 gene (9). The primer used to generate this was 5'-GAACAAGAGTTAGAAATAGGAAAGGT-3' (italicics). This oligonucleotide was used for the gel shift assays not to contain a TATA region. An E. histolytica hgl5 promoter sequence (5'-AATTGCTATACGGGTACCAGCGCTACAAGTGGAATGAG-3') with a mutated GAAC region (bold) was used as double-stranded competitors for the gel shift assay. The probe was purified by a polyacrylamide gel electrophoresis procedure (24). Modifications on this method included the incubation of the polyacrylamide gel section containing the probe at 37 °C overnight in elution buffer (TE (10 mM Tris, 1 mM EDTA, pH 8.0) with 100 mM NaCl) prior to centrifugation for 30 min at 10,000 g. The supernatant was then saved and the pellet washed with an additional 400 μl of elution buffer and re-centrifuged. The two supernatants were combined, the pellet discarded, and the filtered probe was ethanol-precipitated at -70 °C. One pmol of this purified probe was labeled with [α-32P]dATP using the Klenow fragment of DNA polymerase I and purified using a monoclonal antibody to a NuClump column (Stratagene, La Jolla, CA).

The protein-DNA interaction occurred in band shift buffer (10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 0.05% non-fat milk powder (Carnation), 3% glycerol, 0.05 mg of bromphenol blue). To this reaction mixture 0.5 μg of salmon sperm, 25 fmol of DNA probe, and 1.2 μg of nuclear extract were added (25). The reaction was allowed to incubate at room temperature (20 °C) for 1 h prior to the electrophoresis of the reaction mix on a non-denaturing polyacrylamide gel for 2–3 h (26). The gel was then fixed, dried, and quantitated by PhosphorImager analysis.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared by the methods described previously (23). The double-stranded oligonucleotide used for the electrophoretic mobility shift assay was 5'-AACGAGCTGACAGGCAACCAAACCAATGTA-3' (italics). This oligonucleotide was used for the gel shift assays not to contain a TATA region. An E. histolytica hgl5 promoter sequence (5'-AATTGCTATACGGGTACCAGCGCTACAAGTGGAATGAG-3') with a mutated GAAC region (bold) was used as double-stranded competitors for the gel shift assay. The probe was purified by a polyacrylamide gel electrophoresis procedure (24). Modifications on this method included the incubation of the polyacrylamide gel section containing the probe at 37 °C overnight in elution buffer (TE (10 mM Tris, 1 mM EDTA, pH 8.0) with 100 mM NaCl) prior to centrifugation for 30 min at 10,000 g. The supernatant was then saved and the pellet washed with an additional 400 μl of elution buffer and re-centrifuged. The two supernatants were combined, the pellet discarded, and the filtered probe was ethanol-precipitated at -70 °C. One pmol of this purified probe was labeled with [α-32P]dATP using the Klenow fragment of DNA polymerase I and purified using a monoclonal antibody to a NuClump column (Stratagene, La Jolla, CA).

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The schematic depicts the various constructs: wild type core promoter, mutated core promoter GAAC element (A), wild type core promoter with an upstream wild type GAAC (B), mutated core promoter with upstream GAAC element (C), mutated core promoter with upstream inverse orientation GAAC (D), wild type core promoter with downstream GAAC (E), and mutated core promoter with downstream GAAC (F).

to have a variable location between the TATA and Inr regions, we hypothesized that it may function in a position-independent manner to control the rate of gene expression. To test this hypothesis, we performed positional analysis of the GAAC region in the hgl5 gene promoter linked to a luciferase reporter gene. The GAAC region was placed upstream of the TATA region by 22 bases. Alternatively GAAC was placed downstream of the Inr by 10 bases or one helical turn (Fig. 1). These plasmids were electroporated into trophozoites and selected for a decrease in luciferase message to 38% of wild type, as reported previously (Fig. 2). Mutation of the GAAC region in the core promoter resulted in moderate enhancement of message accumulation and luciferase levels were 284% of wild type (Fig. 2E). Insertion of a GAAC region downstream of a wild type core promoter resulted in decreased luciferase message and enzyme levels were 44.5% of wild type (Fig. 2F).

Northern blots were performed with total RNA harvested from the trophozoites (Fig. 2). Luciferase assays were performed on cells stably transfected with various constructs (Fig. 2). Mutation of the GAAC region in the core promoter resulted in a decrease in luciferase message to 38% of wild type, as reported previously (Fig. 2A) (9). Insertion of a wild type GAAC sequence upstream of a mutated core promoter, in either the native or inverse orientation, did not reconstitute luciferase message to wild type levels (Fig. 2, C and D) and in fact resulted in luciferase enzyme activity that was 17 and 7.4% of wild type. Insertion of a wild type GAAC sequence 22 bases upstream of a wild type core promoter also resulted in a marked diminution of reporter gene message levels and luciferase expression was 43% of wild type (Fig. 2B). Insertion of a wild type GAAC sequence downstream of a wild type core promoter resulted in moderate enhancement of message accumulation and luciferase levels were 284% of wild type (Fig. 2E). Insertion of a GAAC region downstream of a mutated core promoter resulted in decreased luciferase message and enzyme levels were 44.5% of wild type (Fig. 2F). Thus, the GAAC element was not able to regulate the level of gene expression in a position-independent manner. In addition, it controls the transcription start site; therefore this sequence does not meet the classical definition of an enhancer element.

**Fig. 1.** Reporter gene constructs for analysis of GAAC function. The schematic depicts the various constructs: wild type core promoter, mutated core promoter GAAC element (A), wild type core promoter with an upstream wild type GAAC (B), mutated core promoter with upstream GAAC element (C), mutated core promoter with upstream inverse orientation GAAC (D), wild type core promoter with downstream GAAC (E), and mutated core promoter with downstream GAAC (F).

**Fig. 2.** Northern blot analysis of reporter gene (luciferase) and control (neomycin) total RNA transcribed from the *E. histolytica* hgl5 promoter constructs containing positional variants of the GAAC element. Total RNA (15 μg) was hybridized with oligonucleotides containing the neomycin (0.8 kilobase) and luciferase (luc) (1.6 kilobase) coding regions. Luciferase enzyme activity relative to the wild type activity is shown below the Northern blot for each construct. The constructs represented are shown in the schematic in Fig. 1. WT, wild type; MUT, mutant.
Thus, this data indicated that the GAAC region was capable of controlling a transcription start site independent of TATA and Inr. In eukaryotes this function had previously been assigned exclusively to the TATA and Inr regions of the core promoter.

An E. histolytica Nuclear Protein Binds to the GAAC Region of the hgl5 Gene in a Sequence-specific Manner—To determine whether the GAAC sequence functioned by interacting with a sequence specific amebic nuclear protein, we utilized EMSA analysis. Amebic nuclear extracts were hybridized with a double-stranded oligonucleotide with the GAAC sequence to identify DNA-protein interactions. EMSA was performed with a probe that contained no TATA region, a mutated and truncated Inr region, and a wild type GAAC region from the hgl5 gene. This oligonucleotide was constructed to prevent DNA-protein interactions with the other core promoter elements, the TATA and Inr.

Incubation of the probe with crude amebic nuclear extract revealed two bands; the lane with probe alone and no amebic nuclear protein had no bands (Fig. 5). Competition experiments were done to determine the specificity of the DNA-protein interaction. The lower band (small arrow) represents a nonspecific DNA-protein interaction as its intensity is not altered by self, unrelated, or mutant competition. Since the oligonucleotide for the EMSA analysis did not contain functional TATA or Inr regions, any specific DNA-protein interaction seen in the EMSA can be ascribed to the GAAC region. Competition assays with self cold unlabeled probe at 2 × and 4 × revealed that a band (Fig. 5, large arrow) was competed by the cold competitor. An unlabeled, unrelated amebic promoter sequence and an oligonucleotide with a mutated GAAC region did not compete this specific band to the same degree. This demonstrated specificity of the DNA-nuclear protein interaction for the oligonucleotide with the GAAC sequence and indicated that the GAAC region specifically recognizes an amebic nuclear protein.

**DISCUSSION**

The major conclusion from this study is that the third core promoter element GAAC (GAACCT) in the hgl5 gene of E. histolytica (i) independently directs a new site of transcription initiation, (ii) controls the rate of transcription initiation, and (iii) interacts in a sequence-specific manner with an amebic nuclear protein (GAAC binding protein(s), GBP(s)). The role of the GAAC region in the hgl5 gene was determined by reporter gene assays, Northern blot analysis, and nuclear run on assays, all of which indicated that the GAAC region controls the rate of transcription.

The GAAC element of the hgl5 gene in E. histolytica is able to control a transcription start site independent of TATA and Inr regions. We demonstrated that positional manipulation of the GAAC region, separated from the TATA and Inr core promoter, resulted in new transcription start sites 2–7 bases downstream of itself. This result occurred consistently with upstream and downstream positioning of the GAAC element and regardless of whether the core promoter region contained a wild type or mutated GAAC sequence. In the context of a wild type core promoter (i.e. wild type TATA, GAAC, and Inr regions) the dominant start site was always in the Inr region regardless of whether a GAAC region was inserted in an upstream or downstream location. However, when the core promoter contained a mutated GAAC sequence, the insertion of a wild type GAAC region in the upstream or downstream location resulted in new transcription start sites that were of equal intensity to that of the wild type Inr site. Thus, in the wild type promoter the interaction between the three regions and the proteins that bind to them are dominant in controlling the transcription initiation site. However, in the context of a mutated GAAC region, this dominance is lost, and new transcrip-
tion initiation sites (directed by the GAAC region) occur. The GAAC sequence was also able to direct a new site of transcription initiation in an inverse orientation, most likely through the creation of a cryptic GAAC site. The identification of a third core promoter element that controls the site of transcription initiation is unprecedented in eukaryotes.

We hypothesized that the GAAC sequence functioned to control the site of transcription initiation via a DNA-protein interaction. The results of the EMSA analysis revealed that an amebic nuclear protein(s), GBP, interacted specifically with the GAAC region. Competition assays with an unrelated hgl5 promoter sequence and an oligonucleotide with a mutated GAAC region pointed to the specificity of this interaction. In the analysis of the EMSA results, it is important to realize that the GAAC-GBP interaction apparently was not dependent on DNA-TBP or DNA-Inr binding protein interactions, since the EMSA probe did not contain functional TATA or Inr regions. The implication is, therefore, that although TBP may require GBP for DNA binding, the GBP does not require TBP or Inr binding protein(s) for accurate and specific DNA binding. Once the amebic GBP and TBP have been isolated, the specific DNA-protein and protein-protein interactions can be characterized in greater detail.

The requirement for a gene or a family of genes to have three core promoter elements is unclear: why would transcription of protein encoding genes in E. histolytica be dependent on a third regulatory region? Perhaps the E. histolytica TFIID complex has multiple DNA binding regions composed of TBP, GBP, and Inr binding proteins. A variety of pre-assembled TFIID complexes could exist, containing some or all of the core promoter-binding proteins. These different TFIID complexes could differentially regulate a variety of core promoters containing all three or only one or two of these regulatory regions.

A second model is based on the fact that TBP in vitro is able to bind to multiple AT-rich sequences (27). It has been shown previously that the specificity of TBP binding to the TATA region is conferred in large part by its proximity to other regulatory regions (28). In an AT-rich organism such as E. histolytica a mechanism may have developed in which a factor such as GBP localizes TBP to the promoter. Thus a model can be hypothesized in which transcription in E. histolytica hgl5 genes may be dependent on protein-protein interactions in which GBP functions to “tether” or localize TBP/TFIID to the core promoter.

Precedence for both these models can be found in the metazoan literature. It has been shown that TFIID can bind multiple regions of the core promoter, including the TATA and Inr regions (29), and Kadonaga has also demonstrated binding of this protein to another core promoter regulatory region, the distal promoter element (13). In support of the second model is
the existence of factors such as TFIIA, which stabilize the binding of TFIIID to the core promoter TATA box (30). Both models provide for a basal complex that has multiple sites to regulate transcription in response to cellular and environmental stimuli.

In conclusion, we have described, for the first time in the metazoan transcription literature, a third core promoter region, GAAC, which is independently able to direct a site of transcription initiation. This sequence from the hgl5 gene of E. histolytica has been shown to interact in a sequence specific manner with an amebic nuclear protein (GBP). The presence of this regulatory core promoter region raises intriguing questions regarding transcriptional control in this primitive protozoan parasite. It is important to consider the possible presence of similar yet to be identified regulatory proteins in other eukaryotes. The isolation of the GAAC-binding protein and characterization of its role in transcriptional control are the next steps in elucidating the role of GBP in the transcriptional machinery of E. histolytica.

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