Comparison of the Post-transcriptional Regulation of the mRNAs for the Surface Proteins PSA (GP46) and MSP (GP63) of Leishmania chagasi*

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MSP (GP63) and PSA (GP46) are abundant 63- and 46-kDa glycolipid-anchored proteins on the surface of the promastigote form of most Leishmania species. MSP is a zinc metalloprotease that confers resistance to host complement-mediated lysis. PSA contains internal repeats of 24 amino acids, and its function is unknown. The steady state levels of mRNAs for both glycoproteins are regulated post-transcriptionally, resulting in about a 30-fold increase as Leishmania chagasi promastigotes grow in vitro from logarithmic phase to stationary phase. Previous studies showed the 3′-untranslated regions (3′-UTRs) of these mRNAs are essential for this post-transcriptional regulation. These two 3′-UTRs of 1.0 and 1.3 kilobases were cloned immediately downstream of a β-galactosidase reporter gene in a plasmid, and segments were systematically deleted to examine which portions of the 3′-UTRs contribute to the post-transcriptional regulation. The 92-nucleotide segment of greatest similarity between the two 3′-UTRs was deleted without loss of regulation, but the segments flanking this similarity region have positive regulatory elements essential for the regulation. We propose that similar, but non-identical, molecular mechanisms regulate the parallel expression of these two L. chagasi mRNAs despite their lack of sequence identity. These post-transcriptional mechanisms resemble the mechanism recently suggested for the regulation of mRNAs encoding the dipeptide (EP) and pentapeptide (GPEET) repeat proteins in Trypanosoma brucei that involves interactions between positive and negative regulatory elements in the 3′-UTR.

Protozoan parasites of the genus Leishmania cause a diverse group of diseases collectively called leishmaniasis that range in severity from spontaneously healing cutaneous ulcers to potentially fatal visceral disease. During their life cycle the Leishmania sp. exist as two developmental stages, i.e. as extracellular promastigotes in the gut of the sandfly vector and as intracellular amastigotes in the phagolysosome of mammalian macrophages. Glycoproteins on the surface of the organism play important roles in its survival in both of these environments. The two best characterized Leishmania surface glycoproteins are the major surface protease (MSP,1 also called GP63 for 63-kDa glycoprotein) and the parasite surface antigen (PSA, also named GP46 for 46-kDa glycoprotein). Although these proteins have historically been called GP63 and GP46, the Nomenclature Working Group for Protozoan Parasites has recommended that protozoan proteins be assigned 3–6-letter names (1), so we will use the nomenclature of MSP and PSA here. Immunization with recombinant versions of either of these proteins or their genes via DNA vaccines provides experimental animals with partial protection against Leishmania challenge (2–6).

Leishmania MSP is a family of closely related zinc metalloproteases that have been found in different reports to (i) confer resistance of promastigotes to complement-mediated lysis (7), (ii) promote attachment to and internalization of promastigotes by host macrophages (8), and (iii) facilitate the intracellular survival of amastigotes in phagolysosomes of host macrophages (9). When virulent promastigotes develop during growth in culture from the less infectious logarithmic phase to the highly infectious stationary form, an 11–30-fold increase in MSP expression occurs (10–12). In Leishmania chagasi, which causes visceral leishmaniasis in Latin America, MSP is encoded by more than 18 genes (MSFs) that fall into three classes on the basis of (i) the stage at which they are expressed in the life cycle and (ii) unique sequences in their 3′-untranslated regions (UTRs) and intergenic regions (IRs) (13). In virulent promastigotes, 3.0-kb MSPS RNAs are expressed in stationary (S) phase but not logarithmic phase of growth, whereas 2.7-kb MSPL RNAs are expressed during logarithmic (L) but not stationary phase. MSPC RNAs of 2.6 and 3.1 kb are constitutively (C) expressed at low levels in both logarithmic and stationary phase (14).

PSA is another family of closely related proteins that have been detected in all Leishmania species examined except for members of the Leishmania braziliensis complex (15–18). All reported nascent PSA sequences contain hydrophobic amino and carboxyl termini that are likely cleaved during translocation of the protein across the endoplasmic reticulum and its linkage to a glycolipid anchor. The function(s) of PSAs is not known, but they possess 3–13 internal leucine-rich repeats of 24 amino acids that have been shown in other proteins to

1 The abbreviations used are: MSP, major surface protease of 63 kDa; PSA, parasite-specific antigen of 46 kDa; UTR, untranslated region; IR, intergenic region from the poly(A) addition site of one gene to the ATG start codon of the downstream gene; βGAL, β-galactosidase; SOE, spliced overlap extension; RFU, relative fluorescence units; olp, overlap region; kb, kilobase(s); S/L ratio, stationary/log ratio; bp, base pair(s); wt, wild type; EP, dipeptide repeat protein; GPEET, pentapeptide repeat protein.

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Plasmid Constructions—The Leishmania expression vector, pXβGAL2, was kindly provided by Stephen Beverley (36). In earlier studies, plasmids were constructed in which the corresponding 3′-UTRs and IR regions of the three MSP gene classes, i.e. MSPS, MSPL, and MSPC, were cloned at the XbaI site downstream of the β-galactosidase gene (Avr II) at the unique Avr II site in pXβGAL2 (36). Since no fragment contains the 5′-UTR and IR of an L. chagasi PSA gene, called PSAA, was isolated by NotI digestion of a genomic DNA phage clone and ligated into a NotI site downstream of the βgal gene in pXβGAL2. Recombinant constructs containing mutations in these 3′-UTRs were initially constructed in pBluescript, after which the mutant 3′-UTRs were gel-purified and cloned downstream of βgal in pXβGAL2. Non-identical sequences in common. The greatest similarity between these three 3′-UTRs is a 92-nucleotide segment with 66% identity. We found, using a β-galactosidase reporter gene, that neither this 92-nucleotide 3′-UTR nor the downstream IR between...
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were quantitated by instant image analyses. 

was followed by 3

right column RNA levels (fold increase in

was cloned downstream of

in which the sequences cloned immediately downstream of

was inserted after

and 39). Southern blots and nuclear run-on assays were used to show that in these cloned stable transfectants the expression remained at a low basal level throughout promastigote growth even though the wild type expression of the

activities and

expression (20, 37). Constructs of

as illustrated schematically. Large open rectangles represent the βGAL-coding region. Large solid rectangles are 3'-UTR sequences, and thin solid rectangles are IR sequences. βGAL activities (RFU/μg of protein) in logarithmic (L) and stationary (S) phase cells were measured and normalized to the activity of a transfectant containing the parent plasmid in the same growth phase (as detailed under "Experimental Procedures"). The ratios of these normalized βGAL activities in stationary versus logarithmic phase transfectants (S/L) were calculated and are shown for each transfectant (left column of numbers). RNAs were isolated from the same transfectants and probed in Northern blots with the βGAL-coding sequence to determine the S/L ratio of steady state RNA levels (right column of numbers). Signals in the Northern blots were quantitated by instant image analyses.

ined their effects on βGAL expression (20, 37). Constructs of the different plasmids were transfected into virulent L. chagasi promastigotes, and cloned transfectants were grown from log-

ate stationary phase (20). In contrast, when

were deleted from each 3'-UTR (Fig. 2B and 3). The region of greatest similarity is

were tested further for regulatory sequence elements (Fig. 2B). A major 92-nucleotide segment of 66% identity called the overlap or olp region. Two other small (<10 bp) regions of limited similarity are located upstream of olp. To see if this similar 92-nucleotide olp segment in each of the two 3'-UTRs contributes to the parallel regulation of their RNAs, the 92 nucleotides were deleted from each 3'-UTR by the SOE PCR technique (see "Experimental Procedures"), and the resultant Δolp 3'-UTR + IR was cloned immediately downstream of βGAL in pXβGAL2 for subsequent stable transfections and βGAL activity measurements.

Fig. 2B shows that deletion of this olp region from these two 3'-UTRs had little if any effect on the regulatory role of either 3'-UTR. The S/L βGAL activity ratio was about 30 ± 5.3 when the 3'-UTRPSAA was present and about 46 ± 16 when the 3'-UTRPSAAΔolp was present, yielding a statistically insignificant p value of 0.153. Likewise, the S/L βGAL ratio was about 63 ± 9 and 59 ± 15 in the presence of the 3'-UTRMSPL and 3'-UTRMSPLΔolp, respectively. Thus, by this targeted deletion analysis it appears the 3'-UTRs do not need the olp segment to up-regulate their RNA levels during growth to stationary phase. This conclusion prompted us to conduct a more systematic deletion analysis of the two 3'-UTRs.

Specific Segments of the 3'-UTRPSAA Are Involved in Gene Regulation—Because deletion of the olp segment did not abrogate logarithmic-stationary gene regulation, we used RNA secondary structure prediction programs to examine the 3'-UTRs of PSAA and MSPL for potential secondary structures that might provide clues about their involvement in regulation. Both 3'-UTRs are about 90% G+C+U, so unfortunately, when both G-C and G-U base pairing are allowed, the number, sizes, and complexities of possible hairpin loops in these 3'-UTRs of 1.0 and 1.3 kb are immense. Thus, these secondary structure analyses were not informative, even when smaller regions of the 3'-UTRs were examined (not shown). Therefore, the two 3'-UTRs were tested further for regulatory sequence elements by deleting ~200-bp segments across the entire 3'-UTR using the SOE PCR technique. In the case of the 3'-UTRPSAA, five adjacent segments were individually deleted (Fig. 3) and the constructs, called AΔ1-AΔ5, were cloned into pXβGAL2 for stable transfection into virulent promastigotes and subsequent analyses (Fig. 4). The right-hand boundary of the deletion in

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construct AΔ5 was designed to occur 13 nucleotides upstream of the polyadenylation site to preserve this site. Point mutations were introduced into SOE PCR primers so that a unique restriction site, AvrII, would be present in recombinant constructs at the site of the deletion. To determine whether changes in βGAL expression were merely due to changes in spacing generated by deletions, the AvrII sites were used to insert non-leishmanial DNA (from pBluescript) of the same length and GC nucleotide content as the 200-bp deletion. This generated another series of recombinant constructs called AΔ1link-AΔ5link. The plasmid deletion constructs and their corresponding linker constructs were stably transfected into promastigotes, and the βGAL activities in logarithmic and stationary phase were analyzed. In every case the βGAL activities derived from a given 200-bp deletion construct and its corresponding “link(ers)” construct were found to be equivalent within experimental error (not shown). Thus, Fig. 4 shows the data for only the deletion constructs.

Deletion AΔ1 caused a 2.7-fold increase in the S/L βGAL ratio compared with the wild type (wt) 3’-UTRPSAA, suggesting there may be a negative control element in this segment. Other deletions had no effect (AΔ2) or caused a decrease (AΔ3, AΔ4, AΔ5) in the S/L ratio. Deletions AΔ3 and AΔ4 exhibited the largest effects, i.e., decreases of 11- and 15-fold in the S/L ratio, respectively. Interestingly, these deleted segments flank olp, whose deletion had no effect (compare Figs. 2 and 4). Thus, sequences deleted in the AΔ3 and AΔ4 constructs possess a positive control element(s) that increases βGAL activity in stationary cells.

To further map putative positive regulatory elements, plasmid constructs AΔ3A and AΔ3B were generated to examine the individual effects of each half of segment 3. In each of these constructs, a non-leishmanial sequence was introduced to preserve the position of the wild type sequence within the 3’-UTR. To our surprise, the presence of either half of segment 3 did not restore wild type βGAL activity in stationary phase (Fig. 4). A similar half-deletion of segment 4 was not constructed.

The locations of the two nucleotide replacements in construct AΔ3 that were used to create the AvrII site (5’-CCTAGG) at the site of the AΔ3 deletion are shown in Fig. 5. Insertion of a 200-bp linker sequence into this site did not significantly change the βGAL S/L ratio from that shown for AΔ3 in Fig. 4 (not shown). Surprisingly, however, re-insertion of the wild type segment 3 sequence into the AvrII site also did not restore the wild type S/L βGAL ratio (Fig. 6, compare AΔ3 and AΔ3wt).

Because the only difference between the AΔ3wt and PSAA 3’-UTR constructs is the AvrII site, we tested whether the AvrII site itself might be responsible for this unexpected result. Three additional constructs were prepared (Fig. 6). To prepare construct AΔ3wt3′, the SOE PCR primers were designed to eliminate the AvrII site on the 5’ side of segment 3 and retain it on the 3’ side. In construct AΔ3wt3′, the AvrII site was eliminated on the 3’ side of segment 3 and retained on the 5’ side. Finally, in construct AΔ3Avr(−), segment 3 was deleted from the 3’-UTRPSAA by SOE PCR without generation of an additional AvrII site. In the resulting stable transfectants, the wild type S/L βGAL ratio was restored in construct AΔ3wt3′ but not in construct AΔ3wt5′ (Fig. 6). Thus, replacement of two
nucleotides to generate an AvrII site at the segment 3-olp boundary of AΔ3wt5' (Fig. 5) is sufficient to cause loss of logarithmic-stationary regulation. However, when segment 3 was deleted without the concomitant insertion of an AvrII site in construct AΔ3Avr(-), the logarithmic-stationary regulation of βGAL was abrogated even more completely than the AΔ3

![Fig. 3. Sequences and locations of the SOE primers used to create deletions in the 3'-UTR](image)

| Mean normalized βGAL S/L | Fold difference vs. wt | p-value |
|---------------------------|-------------------------|---------|
| PS41 3'UTR                |                         |         |
| AΔ1                       | 30.5 ± 11.9             |         |
| AΔ2                       | 81.3 ± 30.3             | 2.7↑    | <.001 |
| AΔ3                       | 42.7 ± 16.5             | 1.4     | .105  |
| AΔ3A                      | 2.70 ± 1.03             | 11.3↓   | <.001 |
| AΔ3B                      | 3.77 ± .17              | 8.7↓    | .010  |
| AΔolp                     | 3.24 ± .74              | 9.4↓    | .009  |
| AΔ4                       | 45.6 ± 27.6             | 1.5     | .155  |
| AΔ5                       | 2.05 ± 24               | 14.9↓   | .001  |
|                           | 9.73 ± 7.38             | 3.1↓    | .006  |
construct (20.6-fold decrease versus 11.3-fold decrease). Thus, the presence of the non-mutated sequence at the 3′-end of segment 3 alone is not sufficient to confer wild type regulation.

Because an AvrII site had also been engineered at the deletion site in the AΔ4 construct (Figs. 4 and 6), the segment 4 wild type sequence was also reinserted into this AvrII site to generate construct AΔ4wt. In this case and in contrast to construct AΔ3wt, the wild type S/L βGal ratio was restored in AΔ4wt (Fig. 6). Thus, the presence of the AvrII site does not appear to alter regulation of βGal expression in the AΔ4 constructs as it does with the AΔ3 construct. Similarly, in experiments not shown, transfectants containing plasmid constructs in which only each end of segment 4 is mutated to an AvrII site have wild type S/L βGal ratios.

Specific Segments of the 3′-UTRPSAA Are Also Involved in Gene Regulation—A deletion analysis of the 1.0-kb 3′-UTRMSFS was also undertaken similar to the 3′-UTRPSAA analysis (Figs. 7 and 8). Four deletion constructs with deleted segments replaced by a single AvrII site, called SΔ1-SΔ4, were cloned into pXβGal2 and stably transfected into virulent promastigotes. Also similar to the PSAA analysis, in each case non-leishmanial DNA (from pBluescript) of the same size and GC content as the deleted segment was cloned into the AvrII site, producing a corresponding set of constructs called SΔ1link-SΔ4link. As was found with the 3′-UTRPSAA, the S/L βGal ratio of a given deletion construct and its corresponding linker construct were found to be the same within experimental error, so only the deletion data are shown (Fig. 8).

Deletion SΔ1 had no effect on the βGal S/L ratio compared with wild type, and SΔ2 resulted in only about a 2-fold change. In contrast, SΔ3 and SΔ4 caused 7.5- and 3.5-fold decreases in the S/L ratio, respectively. Thus, similar to the 3′-UTRPSAA data, deletions of the segments flanking the olp region exerted the largest effects, and in both cases, the deletions caused a decrease of the βGal S/L ratio. Constructs SΔ3A and SΔ3B were also generated to examine the effects of each half of segment 3 (Fig. 8). Similar to the findings with AΔ3A and AΔ3B (Fig. 4), the presence of either half of segment 3 of the 3′-UTRMSFS did not restore the wild type S/L ratio. Thus, the positive regulatory element(s) extends across both halves of segment 3 of the 3′-UTRMSFS as it does in segment 3 of the 3′-UTRPSAA.

Because of the effects of the engineered AvrII site in some of the 3′-UTRPSAA constructs, the wild type segment 3 sequence was reinserted at the AvrII site of construct SΔ3 to generate construct SΔ3wt. Fig. 5 shows the three-nucleotide replacements that were generated during SOE PCR to create this AvrII site. In contrast to AΔ3wt (Figs. 4 and 6), insertion of wild type segment 3 sequence into the AvrII site of SΔ3 restored the wild type S/L βGal ratio (SΔ3wt in Fig. 8). A similar restoration of wild type activity was obtained when the wild type segment 4 sequence was replaced into the AvrII site of SΔ4 (not shown). Thus, the nucleotide replacements used to create the AvrII site in these SΔ constructs do not affect expression of the upstream βGal gene.

Insertion of Region 3 from the 3′-UTRPSAA Did Not Restore Regulation in Construct SΔ3—Because deletions of the segments flanking the olp region in both 3′-UTRMSFS and 3′-UTRPSAA had similar effects, we tested whether these segments in one 3′-UTR could be replaced with the corresponding segments of the other 3′-UTR and still retain the regulation. Therefore, PSAA segment 3 was inserted into the AvrII site of construct SΔ3 to generate construct SΔ3-Awt3 (bottom of Fig. 8). In contrast to SΔ3wt, the wild type S/L βGal ratio of the 3′-UTRMSFS was not restored in SΔ3-Awt3. Instead, the S/L ratio of the original SΔ3 dropped even further, i.e. from 7.5- to 22.2-fold. A similar result was obtained when PSAA segment 4 was inserted into construct SΔ4, i.e. the wild type S/L βGal ratio dropped still further instead of being restored (data not shown). Therefore, in these two examples the regulatory region of one 3′-UTR could not replace the correspondingly positioned regulatory region of the other 3′-UTR, suggesting that these regulatory regions function only within the context of their own 3′-UTRs.

DISCUSSION

The purpose of the current work was to map sequences in the 3′-UTRs of two tandemly repeated gene classes of L. chagasi whose mRNAs are expressed at similar times in the growth cycle of the parasite. We hypothesized that similar molecular features would account for their similar patterns of expression. Our data revealed that the mechanisms regulating levels of MSFS and GP46A RNAs are likely to be complex, involving at least several regions of their 3′-UTRs. Furthermore, our data suggest that different features of each of these 3′-UTR sequences regulate gene expression.

Many differentially expressed trypanosomatid genes are regulated post-transcriptionally by molecular mechanisms involving their 3′-UTRs (21–28, 40–42). The most extensively studied group of post-transcriptionally regulated genes in trypanosomatids is the T. brucei gene family encoding the related acidic repetitive proteins, EP and GPEET (previously called procyclic acidic repetitive protein or PARP (43)), found exclusively on the surface of the procyclic (insect) form of T. brucei (29, 30). The coding regions of the EPs and GPEETs in the T. brucei genome are similar, but their short 3′-UTRs (300 bp) share only a conserved 26-mer sequence. The 100-fold higher steady state level of the EP and GPEET mRNAs in the procyclic form than in the bloodstream form is controlled mainly by elements in their 3′-UTRs (30, 44, 45). Deletion analysis of the EP1 3′-UTR (30, 32) and characterization of its secondary structure by RNase digestion and lead hydrolysis (31) indicate that this 3′-UTR consists of three domains, I, II, and III. The 5′ and 3′ domains I and III, respectively, form independent stem-loop structures in the RNA, whereas the central domain II contains the conserved 26-mer as a single-strand. Domains I and III both have positive regulatory elements, and it has been proposed that in procyclic trypanosomes one or more factors bind to these positive elements in the flanking stem-loops, shielding domain II from endonuclease degradation. In bloodstream trypanosomes, which presumably lack these positive regulators, the single-stranded domain II is exposed to endonuclease activity and quickly degraded (29, 31).

Similar to T. brucei EP and GPEET, Leishmania PSA and MSP are the major surface glycoproteins on the insect form of parasite, and these Leishmania and T. brucei genes are post-transcriptionally regulated in a parallel manner via their dissimilar 3′-UTR sequences. The extent to which the patterns of cis-acting regulatory elements in the 3′-UTRs of their mRNAs resemble each other is intriguing. Similar to the EPs and
GPEETs, PSAA and MSPS are regulated in a parallel fashion by their 3’-UTRs, yet these 3’-UTRs contain little sequence similarity. Likewise, similar to domains I and III of the EP1 3’-UTR, segments 3 and 4 of the PSAA and MSPS 3’-UTRs contain positive regulatory elements that flank a conserved region (domain II in the PSAA and MSPS). In the 3’-UTR of PSAA, deletions of segments 3 and 4 cause a 7.5- and 3.5-fold drop, respectively, in MSPS up-expression in stationary phase (Fig. 8). In both of these Leishmania genes the positive regulatory element(s) in segment 3 could not be further localized by deleting just the 5’ or the 3’ boundary of segment 3. Similarly, the positive regulatory element(s) in domains I and III of the EP1 3’-UTR extend across the stem-loop in most of the domain (30–32). The PSAA and MSPS data also suggest that the regulatory elements in segments 3 and 4 are both necessary, but neither is sufficient to confer full up-regulation of gene expression, again similar to domains I and III of EP1. It is not known whether possible hairpin loops in segments 3 and 4 of PSAA and MSPS play the same roles as the hairpin loops in domains I and III of the EP1 3’-UTR (31). The 200-nucleotide sequences of segments 3 and 4 have potential hairpin loops, as detected by RNA secondary structure prediction programs (not shown), but these sequences are too long for the predictions to be reliable. RNase digestion and lead hydrolysis experiments, similar to those conducted on the EP1 3’-UTR (31), will be necessary to clarify this question.

Unexpectedly, when the sequence of segment 3 was reintroduced into the engineered AvrII site in the A∆3 deletion construct, wild type activity was not restored (Fig. 6). However, when the sequence at the 3’ boundary but not at the 5’ boundary of segment 3 was restored to wild type (i.e. without the

FIG. 6. Additional characterizations of the effect of deletions of segment 3 and segment 4 in the 3’-UTR of PSAA, Plasmids are depicted by schematic diagrams as described in the legend for Fig. 4. The relative positions of AvrII (A) sites are shown. The mean normalized βGAL S/L ratio was calculated as described in the legend for Fig. 4 and under “Experimental Procedures.” For example, there is an 11.3-fold (or 30.5/2.70) decrease in the mean normalized S/L ratio in the A∆3 transfecants compared with wild type. The data shown in Fig. 4 for the wild type construct and constructs A∆3 and A∆Δlp are also shown here for the sake of comparison.

FIG. 7. Sequences and locations of the SOE primers used to create deletions in the 3’-UTR of MSPS. The symbols are the same as indicated in the legend for Fig. 3.
AvrII site), activity was restored to wild type level. Thus, the wild type context at the 3' end of segment 3 is necessary for regulation of PSAA stationary phase expression. However, it is not a sufficient regulatory factor, since wild type regulation was not achieved by the presence of the wild type 3' end of segment 3 alone, as demonstrated by experiments with transfectants containing the AΔAvr(–) deletion construct (Fig. 6). This AΔAvr(–) construct shows that the two altered bases at the 3' end of segment 3 do not substitute for the entire segment 3. Furthermore, the positive effect of segment 3 and its wild type 3' sequence (Figs. 4–6) was active only when the olp sequence was present. When the olp sequence was deleted, AΔOlpl, replacement of the two bases to generate the AvrII site at the 3' end of segment 3 (Fig. 5) did not have the negative effect on regulation that is demonstrated by AΔ3wt and AΔ3wt5' (Fig. 6). These data are consistent with a model in which positive regulation by segment 3 results from shielding a negative element in the olp segment, such as a degradation signal. When the degradation signal is absent, as in the AΔOlpl deletion construct, the protective effect of segment 3 is unnecessary, and wild type regulation is achieved. This is analogous to the involvement of domains I and II in the regulation of EP/GPEET expression.

When segment 4 of 3'-UTRPSAA is deleted, there is a 15-fold loss of regulation that can be restored by reintroduction of wild type sequence into the engineered AvrII site (Fig. 6), again similar in this case to the involvement of domains II and III in the regulation of EP/GPEET expression. Thus, segments 3 and 4 are necessary but not sufficient alone for regulation of PSAA gene expression. To determine whether these segments interact, a construct in which both segments are deleted will be necessary.

In summary, the regulatory effects of the 3'-UTRPSAA and 3'-UTRMSPS are clearly complex and multifaceted. Our data are consistent with a model in which segment 1 of the 3'-UTRPSAA contains a modest negative regulatory element, resulting in a 2.7-fold negative regulatory effect. Segments 3 and 4 each contain positive regulatory elements that appear to shield the olp region. Similarly, the 3'-UTRMSPS contains positive regulatory elements in segments 3 and 4 that flank the olp region, although there does not appear to be a weak negative regulator in segment 1 as there is in the 3'-UTRPSAA. Nucleotides at the boundary between segment 3 and olp appear to be critical for PSAA regulation, but we have no evidence for their involvement in MSPS regulation (Figs. 5, 6, and 8). The AvrII site in the 3'-UTRMSPS constructs was generated by two replacement mutations separated by a single base pair, both of which are in segment 3. The AvrII site in the 3'-UTRMSPS was generated by three replacements, one of which is in segment 3 and two of which are adjacent to each other three base pairs downstream in the olp sequence. Five additional adjacent nucleotides in segment 3 are shared between the 3'-UTRPSAA and 3'-UTRMSPS, as indicated by the diagonal lines in Fig. 5. All three replacements used to generate the AvrII site in the 3'-UTRMSPS disrupt identical nucleotides in the two 3'-UTRs, yet there was only an effect on the regulation of PSAA3'-UTR constructs. It will be worthwhile to determine whether these five nucleotides are important in the regulation conferred by the 3'-UTRPSAA but not 3'-UTRMSPS.

The similar features in the regulation of these Leishmania genes and those of the T. brucei EP/GPEETs support the possibility that there are common themes to the molecular mechanisms determining post-transcriptional regulation of trypanosomatid gene expression through sequences in their 3'-UTRs. Further elucidation of these regulatory mechanisms will require an even more detailed dissection of the 3'-UTR sequences and the potential proteins with which they interact than has been undertaken to date.

**FIG. 8. Segments 3 and 4 of the 3'-UTRMSPS contain positive regulatory elements.** Plasmids are depicted by schematic diagrams as described in the legend for Fig. 4. The mean normalized βGAL S/L ratio was calculated as described in the legend for Fig. 4 and under “Experimental Procedures.” For example, there is a 7.5-fold (or 62.5/8.37) decrease in the mean normalized S/L ratio in the S3 transfectants compared with wild type.
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