Antigenic variation in *Plasmodium falciparum* malaria parasites results from switches in expression among members of the multicopy *var* gene family. This family is subject to allelic exclusion by which particular genes are expressed while the rest of the family remains transcriptionally silent. Evidence from reporter constructs indicates that *var* gene silencing involves a cooperative interaction between the *var* intron and an upstream element and requires transition of the parasites through S-phase of the cell cycle. These findings implicate chromatin assembly in the process of regulating *var* gene expression and antigenic variation. Here we characterize the *var* intron and the elements within it that are necessary for *var* transcriptional silencing. Alignments of *var* introns show a highly conserved structure that consists of three discreet regions with distinct base pair compositions. The middle region is highly AT-rich and is sufficient to silence an associated *var* promoter. Constructs that include a typical *var* intron upstream of a reporter gene or drug-selectable marker reveal that the intron also possesses promoter activity, presumably providing an explanation for the origin of the previously described *var* “sterile” transcripts. Deletions that disable the promoter activity of the intron also eliminate its ability to function as a silencer. These findings suggest that interactions between the regions of these two promoters and the generation of the sterile transcripts play a significant role in regulating *var* gene expression.

The deadliest form of malaria is the result of infection by the protozoan parasite *Plasmodium falciparum*. The disease is characterized by recurrent waves of parasitemia in which each wave represents a population of infected red blood cells with distinct antigenic properties and cytoadherent phenotypes (1). The parasites in these waves use a mechanism of antigenic variation to switch among major variable parasite-produced proteins (PIEMP1) that are placed on the surface of the infected host red blood cells. These switches allow parasites to escape clearance by the host antibody response, leading to persistent infections of variable severity. Antigenic variation thus ensures against the elimination of *P. falciparum* by interfering with the development of complete anti-PIEMP1 immunity.

PIEMP1 is encoded by a multicopy gene family termed *var* (2–4). About 60 different *var* genes are present within the haploid genome of each parasite (3, 5), encoding forms of PIEMP1 with unique antigenic and cytoadherent properties. Switches in expression between individual members of the *var* gene family account for the antigenic variation in a *P. falciparum* population over the course of an infection (4). A mechanism of allelic exclusion appears to control expression of the individual genes (6, 7), thus enabling the parasite population to survive. Without such exclusion, expression of all copies simultaneously would result in premature expenditure of the antigenic repertoire, whereas incomplete silencing of previously expressed copies would lead to clearance by antibodies generated against them. The molecular mechanisms responsible for activating and silencing the genes within this family, and thus coordinating the process of antigenic variation, therefore represent a process imperative for parasite survival.

Although gene duplications, deletions, and recombinations within the family are not uncommon, switches in expression were not found to be accompanied by promoter DNA sequence alterations (8). Silent promoters were shown to be rendered transcriptionally active when removed from the chromosome and placed on transfected episomes (8). These results indicated that switches in expression were probably not the result of changes in transcription factors. Silencing of episomal *var* promoters was restored, however, when a *var* intron was also incorporated into the transfected plasmid (9). Silencing because of the presence of the intron was specific to *var* promoters and depended upon transition of the transfected parasite through S-phase of the cell cycle. These findings associate control of *var* expression with changes in chromatin structure and epigenetic mechanisms of regulation that may have some features in common with chromatin-mediated gene regulation in other organisms.

Members of the *var* gene family are found in tandem arrayed clusters within the internal regions of chromosomes 4, 7, 8, and 12 and as individual genes within subtelomeric regions that also contain genes of the *rifin* and *stevor* families (1, 10). In both internal and subtelomeric regions, transcriptionally active *var* genes have been found adjacent to silent copies, indicating that chromosomal position does not dictate expression. Gene clusters appear to be partitioned into transcriptionally active and silent regions. Chromatin “boundaries” or “insulators” typically separate such regions to prevent the spread of silent and active chromatin structure into adjacent regions and to prevent inappropriate “cross-talk” between the regulatory elements of
FIG. 1. Sequence alignments and nucleotide composition analysis of var introns from the subtelomeric and internal regions of different P. falciparum chromosomes. A, the intron sequences align into three distinct regions with prominent asymmetry in strand composition. Conserved repeats 5'-TG/G(A/T)G/TA-3' are found only in region 1, whereas copies of the complementary repeat 5'-AC/A(T/C)A-3' are found only in region 3 (shaded). Conserved GT and AG dinucleotides at the intron splice boundaries are highlighted in reverse. Conserved repeat elements containing a putative initiator element are boxed, and the ltr-like initiator sequence (TCATA) is shaded. A similar conserved potential initiator element found on the opposite strand is underlined. GenBank™ sequence accession numbers (leading number of each label indicates the assigned chromosome): 1a, AL031747; 2a, AE001366; 2b, AE001434; 3a, AL034560; 3b, AL034559; 4a, AL035477; 7a, L40608; 7b and 7c, L40609; 12a, AC006279; 12b, AC006280. B, histograms showing base pair compositions of the forward strand of the three regions of the intron. Region 1 contains ~20% G and 1% C content in the forward strand, whereas region 3 contains 2% G and 18% C. Contrasts are evident also in the T and A composition of these regions. Region 2 of the forward strand contains >70% A content.
genes in close proximity to one another (11, 12). As a result, DNA elements that function as silencers are frequently found to include boundaries so as not to influence the transcriptional activity of adjacent genes. A number of boundaries and insulators have been identified and studied in several eukaryotic organisms; they vary greatly in their sequence and structure. Several of these elements have been shown to contain binding sites for characterized proteins, including transcription factors or RNA polymerase complexes (11). A few, including the scs and scs’ elements in Drosophila (13) and several elements described in yeast, have been shown to include functional promoters associated with active transcription (14, 15). Exactly how these binding sites and transcriptional activity relate to gene silencing or boundary/insulator activity is not yet fully understood.

Here we describe experiments indicating that each var gene contains two functional promoters, the first upstream of var exon I and the second within the intron. The upstream promoter contains an initiator element at a transcription start site similar to the metazoan Inr sequence that has also been found in Trichomonas vaginalis parasites. Comparative alignments show that var introns exhibit a conserved architecture consisting of three regions differing in base pair composition. Dissection of a typical var intron demonstrates that these regions have different effects on var silencing, particularly the AT-rich central region, which alone is sufficient to silence the upstream var promoter. This central region is also necessary for the promoter activity of the intron, which presumably gives rise to the previously identified “sterile” transcripts associated with the intron and var exon 2 (3, 16). Deletions that remove the promoter activity of the intron also impair its ability to function as a silencer. These results provide the first evidence that the intron promoter and sterile transcripts play a role in var gene regulation and allelic exclusion in P. falciparum.

**EXPERIMENTAL PROCEDURES**

Parasite Cultivation and Gradient Purification—The P. falciparum parasite line Dd2 was cultivated at 5% hematocrit in RPMI 1640 medium supplemented with 0.5% Albumax II (Invitrogen), 0.25% sodium bicarbonate, and 0.1 mg ml⁻¹ gentamicin. The cultures were kept under an atmosphere of 90% nitrogen, 5% oxygen, and 5% carbon dioxide at 37 °C. Percoll/sorbitol gradient purification (17) was performed by laying 3 ml of 40% Percoll/6% sorbitol/RPMI 1640 medium over 3 ml of 70% Percoll/6% sorbitol/RPMI 1640 medium in a 15-ml centrifuge tube. One ml of parasitized erythrocytes at 50% hematocrit was layered on top of the gradient, the gradient was centrifuged at 12,000 g for 20 min at 20 °C, and the cells at the gradient interface were recovered and washed twice with 10 volumes of complete media. Microscopic evaluation of the recovered cells verified that they were over 95% infected with late stage parasites, and these cells were reintroduced into culture.

For drug resistance assays, the parasite line 3D7 was transfected with pVLH/IDH as described above, then cultured in media containing 40 ng/ml pyrimethamine (18). Plasmid rescue experiments were performed by transforming *Escherichia coli*-competent cells with 100 ng of purified *P. falciparum* genomic DNA (19).

Luciferase Expression Constructs—The plasmids pVLH-1 (8) and pVLH/int (9) have been described. The sequence of the intron used in the pVLH/int construct is shown in Fig. 1A (labeled 2b). Various portions of this intron were amplified by using specific primers that had been modified to include a BamHI site on the 5’ end and a NotI site on the 3’ end. Such primers were used to amplify region 2 (bp 141–712 of the intron), region 3 (bp 687–872), regions 1 and 2 (bp 141–712), and regions 2 and 3 (bp 141–762). These fragments were cloned into BamHI/NotI-digested pVLH-1 to create the plasmids pVLH/intΔ1Δ3, pVLH/intΔ1Δ2, pVLH/intΔ2Δ3, and pVLH/intΔ1Δ2Δ3, respectively, where the Δ refers to the deleted intron region(s). pVLH/intΔ2Δ3 was created by cutting...
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out region 1 of the intron from PVLIH/1 by restriction digestion with BamHI/DreI and cloning this fragment directly into BamHI/SmaI-digested PVLIH-1. PVLIH/1 were the result of a spontaneous deletion in PVLIH/1 that occurred while propagating the plasmid in E. coli. This plasmid was sequenced and found to be missing bp 161–688 of the intron. The plasmid PVLIH-1 was made by digesting PVLIH/1 with SstI and Smal. The intron fragment was then cloned upstream of the luciferase coding region of similarly digested PGL2-basic (Promega). The simian virus 40 polyadenylation site of PGL2-basic was then replaced with a BamHI/ClaI fragment containing the hpr2 3' sequence from the plasmid PVLIH-1. The various intron fragments were also cloned into BamHI/NolI-digested PVLIH-1 to generate plasmids in which the individual portions of the intron replaced the full-length intron to drive luciferase expression. Site-directed mutagenesis was performed by using the QuikChange mutagenesis kit (Stratagene) and the manufacturer’s protocols.

Parasite Transfection and Luciferase Assays—Parasites were transfected as described (20). Briefly, uninfected human erythrocytes were electroporated in the presence of 50 μg of plasmid DNA in incomplete cytomix using a Bio-Rad gene pulser, 0.2-cm cuvettes, and conditions of 0.21 kV and 360 microfarads. Erythrocytes from such electroporations were combined for each 5-ml culture and inoculated with late stage parasites purified using a Percoll/sorbitol gradient. Luciferase assays were performed on 5-ml cultures at selected times after transfection (9). Error bars in graphs represent standard deviations of experiments done in triplicate.

Previous work has shown that plasmids containing an intron require passage through S-phase to silence an upstream var promoter (9). However, because var promoters are only active during the ring stage (prior to S-phase), silencing is not detected after transfection until the ring stage of the following cycle, after which silencing is maintained through subsequent cell cycles. All transfection experiments described in this manuscript were performed by allowing parasites to invade DNA-loaded erythrocytes (20) and then separating the parasites from the DNA-loaded erythrocytes by Percoll/sorbitol gradients. Return of the purified parasites to culture with fresh, unloaded erythrocytes thus ensured that all transfected plasmids had been through at least one S-phase. Activity of the var7b promoter was always measured in ring-stage parasites unless otherwise stated.

RESULTS

Analysis of var Intron Structure—Typical var genes contain two exons separated by a 0.8–1.2 kb intron (3). Fig. 1A shows an alignment of the sequences of introns from 11 different var sequences in the GenBank™ data base. These sequences are from several different parasite lines and include genes from both subtelomeric and internal regions of chromosomes. All show similar structure with three distinct regions that are distinguished by their sequence similarity and nucleotide composition (Fig. 1A). Several repeat sequence elements occur in these regions, including TGTATGTG in region 1 and the direct complement of this sequence (ACATACAC) in region 3.

Forward strand composition data, summarized by the histograms in Fig. 1B, show prominent asymmetry of the G versus C and A versus T contents of regions 1 and 3. The forward strand of region 2 consists of over 70% A with very little G or C. The differences among regions 1, 2, and 3 were found to be distinct without ambiguity of their borders in the alignments. Identification of an INR Element in a var Promoter—Primer extension was previously performed by using mRNA from an expressed internal gene (var7b), and the transcription start site was mapped to a conserved A residue at the center position within the sequence TCATA (8). Interestingly, this 5-base pair sequence was recently identified as a promoter element of numerous genes in the parasite T. vaginalis (21). In all cases, the A at position 3 was the transcription start point, and certain specific point mutations within the sequence resulted in substantially reduced promoter activity. This element is very similar in function and sequence to a metazoan initiator element (Inr), suggesting that these elements were present in an ancestor early in eukaryotic evolution.

To test whether the TCATA sequence found at the transcrip-
To further characterize the intron promoter and to determine what portion or portions were necessary for promoter activity, we transfected parasites with constructs that included either the entire intron 2b in forward or reverse orientation, region 2 alone, or intron 2b with region 2 deleted. To determine the relative strength of the intron promoter, we compared the luciferase activity of these constructs to that of pVLH-1. Late trophozoites containing reporter constructs driven by intron 2b and ring-stage parasites transfected with pVLH-1 were assayed to allow a direct comparison of the peak activity of both promoters. The results (Fig. 4A) show that the intron can function as a promoter in either orientation, a characteristic previously described for several TATA-less promoters in other organisms (29–31). Further, the luciferase levels from the various constructs show that intron region 2, the region that includes putative Inr elements similar to that in the upstream var promoter, is necessary for intron promoter activity and is sufficient on its own for luciferase expression.

Constructs containing the intron 2b promoter gave significantly lower luciferase activity than those containing the var7b promoter (Fig. 4A). To determine whether this low level of promoter activity was indicative of a truly functional promoter and was not simply spurious, the plasmid pVLH/int was modified to include the coding region of the human dhfr gene downstream of intron 2b (Fig. 4B). In this construct (pVLH/IDH), the intron acts as a silencer of the upstream var7b promoter and as a promoter driving expression of the dhfr gene. As expected, parasites transfected with this construct demonstrated silencing of the luciferase gene similar to that seen with pVLH/int (data not shown). When grown in media containing pyrimethamine, drug resistant parasites were selected after 3 weeks of drug pressure, as is typical for parasites transfected with plasmids containing drug resistance markers. Plasmid rescue experiments showed that the resistant parasites were carrying the construct pVLH/IDH. The strong repression of luciferase expression indicates that dhfr expression is unlikely to be the result of “read-through” transcription initiating from the var7b promoter. In addition, control cultures of untransfected parasites or parasites transfected with a modified pVLH/IDH where the intron had been deleted failed to select any drug-resistant parasites. These results indicate that the promoter activity of the intron was sufficient to alter the phenotype of the parasite and, combined with previously published Northern blots that show significant transcription of the “sterile” transcripts (3, 16), strongly support the conclusion that var introns are functionally active promoters in P. falciparum.
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Relative Effects of Intron Regions 1, 2, and 3 on var Gene Silencing—To explore the role of intron regions 1, 2, and 3 on var gene silencing, we constructed modified pVLH/int plasmids in which the full-length intron 2b was replaced by each individual region separately and in combination with one another. Luciferase expression levels from transfected parasites were then measured to determine the effects of the various regions of the intron on silencing of the associated var7b promoter. The expression assays were performed on ring stages after all the transfected parasites had passed through S-phase.

All constructs that included region 2, including constructs where region 2 was the only portion of the intron included, showed levels of var promoter silencing equivalent to that obtained from the complete intron (Fig. 5A). Constructs that included only regions 1 or 3, or both regions 1 and 3 but excluding region 2, showed partial repression of luciferase expression (50–70%) but were not able to fully silence var7b promoter activity. To further demonstrate association between the intron’s promoter activity and its ability to function as a silencing element, each combination of intron 2b fragments was placed upstream of luciferase to test for promoter activity (Fig. 5B). These experiments show a direct association between the intron’s promoter activity and its ability to silence the associated var promoter. In particular, these results highlight the importance of region 2 for both the silencing ability of the intron as well as its inherent promoter activity.

DISCUSSION

Silencing and selective expression of var genes have vital roles in the antigenic variation that is central to the pathogenesis of P. falciparum malaria. Models for var transcriptional control, however, are undeveloped, and only limited analogies can be drawn to the regulatory mechanisms of multigene families in other eukaryotes. Systems that show some analogies (for example, the vsg genes of African trypanosomes (32), the usp genes of Giardia (33), and the odor receptor genes of mammals (34)) are controlled by mechanisms that remain unclear and appear to differ considerably from those in P. falciparum. A mechanism in which regions containing two separate promoters co-operate to control gene silencing has not been described for these other multigene families and may be unique to var genes.

Reports differ on the number of var genes expressed at one time in a single parasite. Nuclear run-on experiments using parasites selected for their cytoadherence phenotype showed a single var gene transcribed in trophozoites (7). Single-cell reverse transcriptase-PCR experiments identified transcripts from multiple var genes in early ring-stage parasites, but only a single transcript remained in trophozoites (6). Detection of only a single transcript band on RNA blots is consistent with proposals that multiple transcripts in early rings are low level and transient, possibly because they are truncated or unstable (24). In contrast, other experiments using single-cell reverse transcriptase-PCR have described multiple var transcripts in trophozoites, suggesting that more than one var gene can be transcribed at this stage of the cell cycle (35). Nevertheless, it is clear that progression through the cell cycle has an important effect on the regulation and detection of var gene transcripts. One manifestation of this effect is the co-operative S-phase-dependent silencing of episomal constructs carrying var promoters in cis with var introns. The transcription from var introns occurs later in the cell cycle than the ring-stage peak of upstream var promoter activity and may relate to the S-phase dependence of silencing.

A function or explanation for the existence of the var sterile transcripts has been lacking since they were first described along with the discovery of the var gene family (3). Recent work on chromatin-mediated gene silencing in other organisms may provide some suggestions. The boundary elements scs and ses' in Drosophila (13) and several boundaries described in yeast (14) are actively transcribed. Promoter activities found in these boundaries are important for their ability to prevent the spread

![Figure 5](image-url)
of silent chromatin outside the silenced locus (36). It has also been shown that several yeast transcription factors can act as boundaries when bound to DNA, suggesting that certain promoters may behave as insulators and partition genomes into functionally independent domains (14, 15). This behavior is pronounced in the human β-globin locus where non-coding transcripts precisely delineate transcriptionally active and silent domains (37). Similar to the transcripts from the var intron, the β-globin non-coding transcripts are expressed during early S-phase at the time when the globin locus is replicated and chromatin assembly and modification occurs. Disruption of the production of these transcripts interferes with chromatin remodeling and proper gene regulation. Thus, the promoter activities of var introns could be functioning in a manner that likewise separates the transcriptionally active and silent chromatin domains of the chromosome neighborhoods in which var genes reside. Alternatively, the intron promoter could function directly in silencing of the upstream var promoter through promoter competition. This has also been proposed as a model for regulating expression of the β-globin locus in vertebrates (38–40). In this system, cis-linked promoters compete for a single shared enhancer, with a developmental switch in expression resulting from one promoter out-competing the other. An effect of promoter competition has recently been described for an insulator element in Drosophila (41). If such a mechanism applies to var genes, both promoters would be competing for a single enhancer element, and silencing of the upstream var promoter would be accompanied by the corresponding up-regulation of the intron promoter. This model would explain the apparent weakness of the intron promoter in which the construct pILH-1 where it has been separated from any var genes reside. Alternatively, sequences with such asymmetric compositions may behave as insulators and partition genomes into separate loops of chromatin representing different domains (48, 50). Such a role would be consistent with a model in which the intron serves as both a silencer and a boundary element that separates each var gene from the neighboring chromatin environment.

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